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The importance of ubiquitin in the trafficking of the insulin responsive glucose transporter GLUT4

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BSc

Submitted in fulfilment of the requirement for the degree of *Master of Science*

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Abstract

The regulation of blood glucose levels in humans, in response to insulin, is essential to survival. This response is mediated through the insulin responsive glucose transporter GLUT4. In response to insulin stimulation GLUT4 is trafficked from intracellular insulin sensitive stores (GSVs GLUT4 storage vesicles) to the plasma membrane of fat and muscle cells allowing uptake of glucose into these cells and lowering of plasma glucose levels. Previous work from our lab has identified that ubiquitination and subsequent deubiquitination of GLUT4 is required for entry and stability in GSVs. This balance of ubiquitination and deubiquitination in mammalian cells is carried out by E3 ligases and deubiquitinating enzymes (DUBs). It appears that E3 ligases allow for targeted entry of GLUT4 into insulin sensitive GSVs and that the DUB USP25 is required for GLUT4 to stably maintained in these GSVs. Using a model developed in our lab my thesis looked at key steps of ubiquitination and deubiquitination to try and identify the E3 ligases and deubiquinases essential for entry and stability of GLUT4 into GLUT4 storage vesicles.

Previous work in our lab identified that knockdown of the ubiquitin specific protease USP25 resulted in a reduction in GLUT4 levels in 3T3-L1 adipocytes. In my thesis I looked at using an inducible retro viral system to identify if the catalytic activity of USP25 was required to maintain GLUT4 expression levels. This method had limited success as I was unable to produce a virus that would always express under induction.

Previous work in our lab had also identified that GLUT4 was ubiquitinated in yeast and in 3T3-L1 adipocytes. BTrCP had previously been identified as a possible E3 ligase that interacts with GLUT4 and so I used this as a candidate ligase. To identify if BTrCP played a role in mediating insulin responsive glucose uptake I utilised siRNA knockdown of BTrCP in 3T3-L1 adipocytes alongside glucose uptake assays. Although knockdown of BTrCP showed no effect on GLUT4 levels in 3T3-L1 adipocytes it did show a decrease in insulin responsive glucose uptake.

These data did not provide any further information on the relationship between GLUT4 and USP25. These data do however indicate that BTrCP does interact in

some way with insulin responsive glucose uptake in 3T3-L1 adipocytes but it is still unclear if this is due to any interaction with GLUT4.

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Author's declaration

I declare that the work presented in this submission has been carried out by myself unless otherwise stated. It is entirely of my own composition and has not been submitted, in whole or in part, for any other degree.

Iain Stewart Adamson

March 2015

Abbreviations

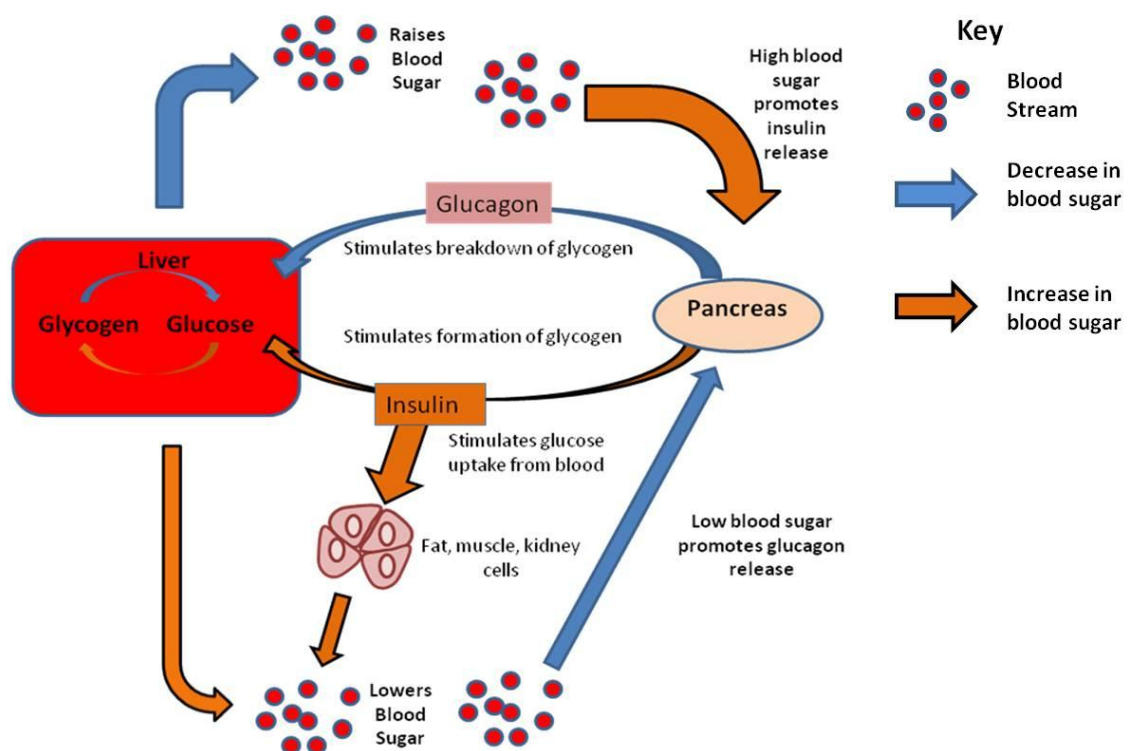
~ Approximately
 ADP adenosine diphosphate
 ARF ADP ribosylation factor
 ATP adenosine triphosphate
 BSA bovine serum albumin
 BTrCP Beta-transducin repeat containing E3 ubiquitin ligase
 ° C degrees Celsius
 Ci curie
 C-terminal carboxy terminal
 ddH₂O double distilled water
 DMEM Dulbecco's modified Eagle's medium
 DMSO dimethyl sulphoxide
 DNA deoxyribonucleic acid
 dNTP deoxynucleotide triphosphate
 DUB deubiquitinating enzyme
 E1 ubiquitin activating enzyme
 E2 ubiquitin conjugating enzyme
 E3 ubiquitin ligase
 E. coli Escherichia coli
 ECL enhanced chemiluminescence
 EDTA ethylenediamine tetraacetic acid
 ER endoplasmic reticulum
 FCS foetal calf serum
 g gram
 xg multiple of gravitational force
 GAE γ adaptin ear
 GAT GGA and TOM1
 GFP *Aequorea victoria* green fluorescent protein
 GGA Golgi localised, γ adaptin ear containing, ARF binding protein
 GLUT glucose transporter
 GST glutathione-S-transferase
 GSV GLUT4 storage vesicle
 HA influenza haemagglutinin epitope tag
 HCl hydrochloric acid
 HEPES 2[4(2-Hydroxyethyl)-1-piperazine]ethanesulphonic acid
 Ig immunoglobulin isotype
 IPTG isopropyl- β -D-thiogalactopyranoside
 IRAP insulin responsive aminopeptidase
 IRS insulin receptor substrate
 IRV insulin responsive vesicle
 JAMM JAB1, MPN/MOV34 metalloenzymes
 k kilo (prefix)
 KCl potassium chloride
 K₂HPO₄ dipotassium hydrogen orthophosphate
 KH₂PO₄ potassium dihydrogen orthophosphate
 l litre
 LSB Laemmli's sample buffer
 μ micro (prefix)
 m milli (prefix)

M molar
MgSO₄ magnesium sulphate
min minute
n nano (prefix)
N-terminal Amino-terminal
NaCl sodium chloride
Na₂HPO₄ disodium hydrogen orthophosphate
NaH₂PO₄ sodium dihydrogen orthophosphate
NaOH sodium hydroxide
NCS newborn calf serum
NEM N-ethyl maleimide
OD₆₀₀ optical density at 600 nm
PAGE polyacrylamide gel electrophoresis
PBS phosphate buffered saline
PBS-T 0.1 % Tween-20 in phosphate buffered saline
PCR polymerase chain reaction
RNA ribonucleic acid
RS retention sequence
SDM site directed mutagenesis
SDS sodium dodecyl sulphate
siRNA short interfering RNA
TAE tris-acetic acid-EDTA
TE tris-EDTA
TfR transferrin receptor
TGN *trans*-Golgi network
Ub ubiquitin
v/v units per unit volume
w/v units weight per unit volume
YT yeast extract, tryptone, NaCl

1 Introduction

1.1 Diabetes

Diabetes mellitus is a disease characterised by persistent hyperglycemia (Zimmet et al. 2001). Normal blood glucose levels lie in the range of between 4 and 7mM rising as high as 10mM after a carbohydrate rich meal (Zimmet et al. 2001). Glucose adsorbed in the intestine enters the blood stream and is detected at the β -cells of the pancreas (Zimmet et al. 2001). This increase in blood glucose levels causes secretion of insulin from pancreatic β -cells, which acts on adipose and muscle tissue to increase uptake of glucose from the blood and inhibits glucose production in the liver (Sesti 2006). This system is shown in figure 1.1.



1.1 A diagrammatic representation of glucose homeostasis in the human body.

Increases in blood glucose levels stimulate insulin release and subsequent lowering of blood glucose levels (orange arrows). Decreases in blood glucose levels stimulate glucagon release and raises blood glucose levels blue arrows. Red spots represent the human blood stream.

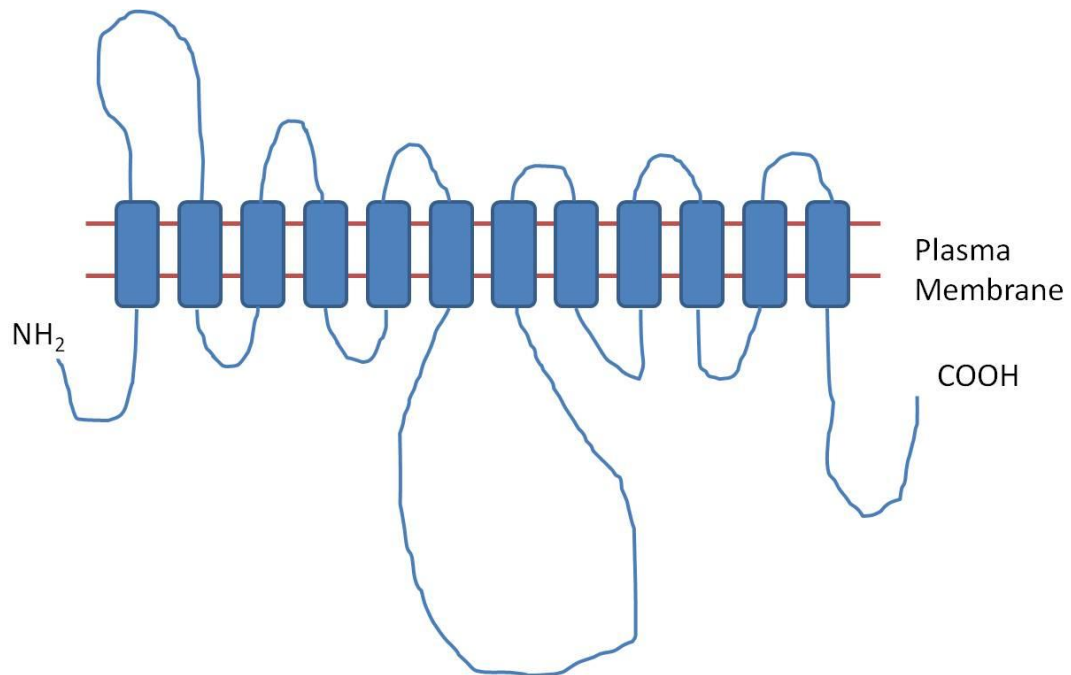
The persistent hyperglycemia characteristic of diabetes is due to no insulin production found in type-1 diabetes, or insulin resistance typical of type-2 diabetes. Type-1 diabetes or insulin dependent diabetes is a result of

autoimmune destruction of β -cell islets, leading to failure of insulin secretion or production (Saltiel & Kahn 2001). Type-1 diabetes can be treated with insulin injections used to control blood glucose levels. Type-2 diabetes is characterised by an underlying resistance to insulin. In insulin-resistance insulin fails to lower plasma glucose levels due to a loss of responsiveness of fat and/or muscle. Persistently high blood glucose levels can lead to renal failure, vascular and cardiac disease and blindness (Saltiel & Kahn 2001). Thus it is important blood glucose levels can be maintained at normal levels.

In recent decades there has been a massive increase in the numbers of people with diabetes with estimates that more than 220 million people worldwide suffer from diabetes (Zimmet et al. 2001). Type-1 diabetes makes up less than 10% of the population living with diabetes, with type-2 diabetes being the most prevalent. The increasing number of individuals needing treatment for type-2 diabetes is putting a strain on health services due to increased prescription of insulin sensitising drugs. With secondary symptoms leading to type-2 individuals requiring time off from the workplace this also makes it a large financial problem. With epidemic levels of type-2 diabetes worldwide it has been an important area of research for the last decade and with estimates of increasing numbers of sufferers of type-2 diabetes, it will remain to be an important area of study for the foreseeable future.

1.2 GLUT4

GLUT4 belongs to a family of facilitative sugar transporters that are predicted to contain 12 membrane spanning domains with both their N and C terminus located intracellularly (Reviewed (Bryant et al. 2002; Watson et al. 2004). Based on their sequences and structural similarities these transporters have been separated into 3 subclasses of sugar transporters with different substrate specificity and tissue distribution. GLUT 1-4 are class I glucose transporters and their predicated topology is shown in figure 1.2 (Bryant et al. 2002).



1.2 Schematic representation of the topology of the class I GLUT family transport proteins.

Within the class I glucose transporters, the different tissue distributions, various K_m values and cellular localisation allow for regulation of glucose transport into different tissues under different physiological conditions. GLUT 1 is expressed ubiquitously at the plasma membrane and is highly expressed in the brain and in erythrocytes (Reviewed (Watson et al. 2004)). This distribution along with a K_m below physiological glucose concentrations highlights GLUT1 as providing a constant level of glucose transport to maintain basal cellular function within all tissues. GLUT4 is highly expressed in skeletal muscle and adipose tissue; under basal conditions GLUT4 is located in intracellular stores. GLUT4 becomes rapidly localised to the plasma membrane of skeletal muscle during exercise and to the plasma membrane of skeletal muscle and adipose tissue upon insulin stimulation (Bryant et al. 2002; Watson et al. 2004). This allows lowering of blood glucose levels after a meal and increased glucose transport to meet the additional energy needs of active muscle (Bryant et al. 2002). Translocation from intracellular stores to the plasma membrane allows for this regulation of glucose transport into fat and muscle cells. Even with 30 years of research since GLUT 4 was identified as the insulin sensitive glucose transporter we do not fully understand all of the trafficking events involved in GLUT4 translocation to the plasma membrane. For example how is GLUT4 targeted to the insulin sensitive compartment and what signals GLUT4 to translocate to the cell membrane in response to insulin? [Bryant et al., 2002].

1.3 GLUT4 storage vesicles

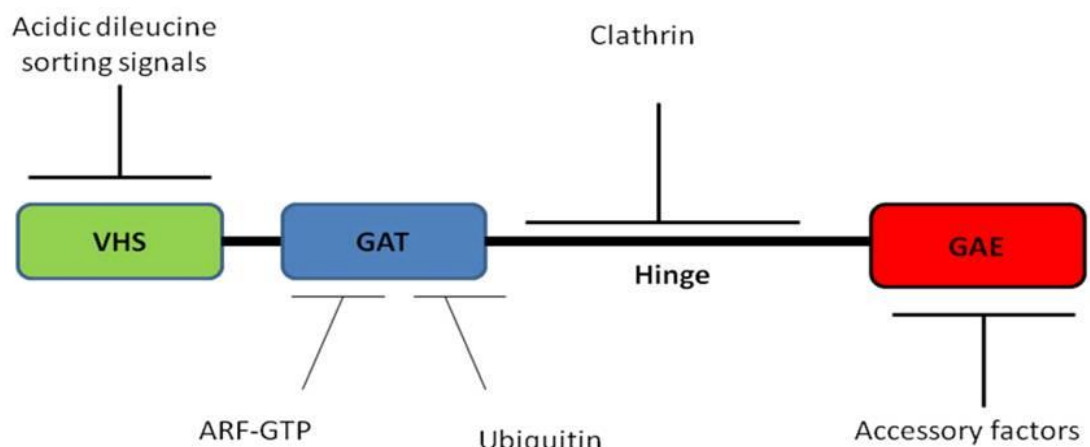
GLUT4 storage vesicles are a population of subcellular compartments. The main identifying feature of GSVs characterised in adipocytes is the lack of a transmembrane protein cellugyrin (Jedrychowski et al. 2010; Kupriyanova et al. 2002). Previous work showed that GLUT4 and cellugyrin co-localised (Kupriyanova & Kandror 2000) but this population did not translocate to the cell surface in response to insulin. Many of the other proteins present in GSVs have also been identified, the vesicle associated membrane protein 2 (VAMP2)(Cain et al. 1992; Martin et al. 1996), insulin responsive aminopeptidase (IRAP)(Ross et al. 1997a), low density liposome receptor related protein1(Jedrychowski et al. 2010) and sortilin(Li et al. 2009).

Sortilin is a 110kDa glycoprotein that has been identified as essential for the formation of GSVs (Lin et al. 1997; Morris et al. 1998). Sortilin expression and formation of GSVs has been shown to occur 2 days post differentiation in 3T3-L1 adipocytes (Shi & Kandror 2005). shRNA knockdown of sortilin results in reduced formation of GSVs in an in vitro budding assay. Further investigation also identified a reduction in insulin stimulated glucose transport (Shi & Kandror 2005). Myc tagged GLUT4 expressed on its own in 3t3-L1 fibroblasts resulted in rapid degradation of the protein. Co-expression with sortilin stabilised the 7myc-GLUT4 and allowed formation of GSVs in non differentiated 3t3-L1 cells shown by an increase in insulin responsive glucose uptake (Shi & Kandror 2005). Yeast two-hybrid and cross linking analysis has identified an interaction between the luminal domain of sortilin with those of both IRAP and GLUT 4 (Shi & Kandror 2007). Formation of the insulin sensitive compartment is dependent upon these luminal interactions. Expression of a truncated sortilin mutant resulted in only partial reconstitution of the insulin responsive compartment in undifferentiated 3T3-L1 adipocytes(Shi et al. 2008; Shi & Kandror 2005).

As noted above LRP1 has been identified as a cargo component of GSVs (Jedrychowski et al. 2010), and has been shown to also play a role in GSV formation and stability. Cross linking analysis in rat epididymal adipocytes identified that LRP1 interacts with sortilin, IRAP and GLUT4. Furthermore shRNA knockdown of LRP1 results in reduced levels of all three proteins (Jedrychowski et al. 2010).

1.4 GGA proteins

One important stage in GLUT4 trafficking is sorting into GSVs. The GGA family of proteins have been implicated in this process. The GGA (golgi localised, gamma adaptin ear containing, Arf binding) proteins were identified as a family of clathrin adapter proteins. There are two GGAs in yeast (*gga1/2*) and three in humans (GGA1,2 and 3). The GGA proteins are comprised of 4 domains; a VHS (Vps 27, Hrs, STAM) domain which binds dileucine sorting motifs, a GAT (GGA and TOM1) domain capable of binding Arf-GTP and ubiquitin, the hinge region that binds clathrin and the GAE (gamma adaptin ear like domain that interacts with accessory proteins (Pelham 2004; Bonifacino 2004).



1.3 Schematic showing domain structure of a typical GGA protein. Adapted from (Pelham 2004)

GGA proteins have been shown to be involved in delivery of cargo proteins from the TGN into the endosomal system through the GGA interaction with ubiquitin moieties (Scott et al. 2004). The GAT domain of GGA3 has been shown to bind to ubiquitin (Puertollano & Bonifacino 2004; Scott et al. 2004). Scott et al 2004 identified that GGAs were essential for sorting Gap1p directly from the TGN to endosomes in yeast. Further experiments in yeast revealed that *ggaΔ* cells grown on a poor nitrogen source and switched to a higher nitrogen containing growth medium did not sort the Gap1p transporter for degradation (Scott et al. 2004). This sorting defect was also maintained when ubiquitin binding portions of the GAT domain are deleted, even though Gap1p is correctly ubiquitinated it

is not sorted to endosomes (Scott et al. 2004). (Kawasaki et al. 2005) also identified that mutation of E250 and D258 of the GAT domain reduces *in vitro* binding of ubiquitin to the GAT domain of GGA3.

In mammalian cells GGA3 has been identified as playing a role in sorting the EGF receptor (Puertollano & Bonifacino 2004). siRNA depletion of GGA3 causes the EGF receptor and the cation independent mannose-6 phosphate receptor to accumulate from the TGN and late endosomes to large early endosomes.

This evidence highlights the interaction between GGAs and ubiquitin as a sorting signal for membrane proteins.

1.5 Gap1p in *Saccharomyces cerevisiae*

The yeast general amino acid permease Gap1p is a twelve transmembrane channel protein that is regulated in response to the nitrogen source on which the yeast are grown (Roberg et al. 1997). When grown on poor nitrogen sources where amino acid uptake is essential Gap1p is trafficked to the plasma membrane. However when grown on rich nitrogen sources such as glutamate Gap1p is trafficked to the endosomal system where it is eventually degraded at the vacuole (Roberg et al. 1997).

Work investigating these alternate fates of Gap1p has identified that different ubiquitin modifications on Gap1p direct these different trafficking events. Monoubiquitination of Gap1p has been shown to result in internalisation of Gap1p from the plasma membrane (Risinger & Kaiser 2008). When Gap1p is polyubiquitinated this has been shown to result in trafficking directly to the vacuole (Risinger & Kaiser 2008). This clearly indicates that membrane protein trafficking can be altered by external stimuli and the ubiquitin state of that protein.

1.6 GLUT4 is ubiquitinated and deubiquitinated

In the absence of insulin, GLUT4 is retained intracellularly, continually cycling through the trans-Golgi network (TGN), endosomal system and GLUT4 storage vesicles GSVs (Bryant et al. 2002). GSVs are defined as the compartment from

where GLUT4 is recruited to the plasma membrane in response to insulin. Previous work in looking at the trafficking of GLUT4 showed that in *S. cerevisiae* it was trafficked in a similar manner to that of Gap1p as detailed in section 1.5. Work carried out on 3T3-L1 adipocytes shows the GLUT4 transporter is ubiquitinated, furthermore trafficking of GLUT4 through the endosomal system to GSVs appears to be ubiquitin dependent as a mutant form of GLUT4 with all 7 cytosolically exposed lysines fails to traffic to the cell surface in response to insulin in 3T3-L1 adipocytes (Lamb et al. 2010). Restoration of ubiquitination to the 7K/R version of GLUT restores insulin responsiveness, however introduction of a constitutively ubiquitinated form of GLUT4 does not show an insulin responsive phenotype indicating that ubiquitination alone does not confer insulin sensitivity. This is also supported by data that shows only approximately 0.1% of GLUT4 is ubiquitinated at steady state (Lamb et al. 2010).

As previously noted to prevent ubiquitinated proteins from being degraded there is a requirement for a deubiquitinating enzyme to remove ubiquitin molecules. There are no DUBs known to be associated with GSVs however there does appear to be an association through a binding partner of the GSV cargo protein IRAP. IRAP has been found to traffic in a similar manner to GLUT4 in adipocytes (Ross et al., 1997). Work using siRNA depletion of IRAP showed a reduction in insulin stimulated GLUT4 translocation (Yeh et al. 2007). An interacting partner of IRAP is the poly-ADP ribose polymerase (PARP) Tankyrase (Sbodio et al. 2002). An interacting partner of Tankyrase is the ubiquitin specific protease USP25, siRNA mediated knockdown of USP25 carried out in our lab has revealed that USP25 depletion results in a reduction of GLUT4 levels in 3T3-L1 adipocytes and a reduction in glucose uptake in response to insulin. These data suggest that USP25 may play a role in maintaining GLUT4 stability in GSVs by removing ubiquitin from GLUT4 molecules contained in GSVs and preventing their degradation.

1.7 Ubiquitination

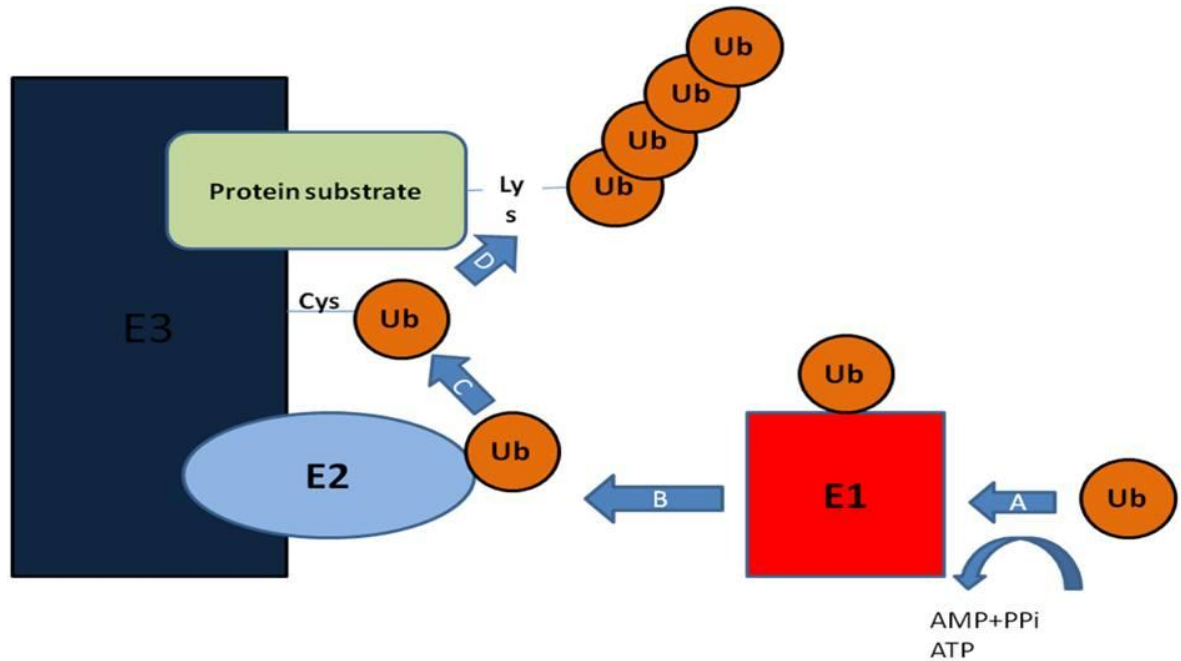
As previously noted GGA proteins interact with ubiquitinated proteins and could therefore be involved in GLUT4 sorting. Post translational modification of proteins occurs after their production and can include phosphorylation, lipidation and conjugation to other proteins. Modification like this can act to

alter a proteins activity, localisation within a cell in a manner similar to the way internal trafficking motifs can affect sorting of the protein.

Ubiquitin is a 76 amino acid polypeptide that acts as a post translational modification and was originally identified as APF-1 (ATP-dependent proteolysis factor 1) a protein of 8.5kD required for proteolysis, which could reversibly conjugate to protein substrates (reviewed(Ciechanover 2013)).

Ubiquitin is conjugated to other proteins by forming an isopeptide bond between a lysine residue on the target protein and a glycine residue on the ubiquitin (Hicke & Dunn 2003). Ubiquitin can become bound at one or multiple sites on a target protein and can also conjugate with itself forming polyubiquitin chains. Depending on the type of modification that occurs, soluble proteins can be degraded by the proteosome and membrane proteins can be trafficked through the endosomal system leading towards lysosomal degradation(Ciechanover 2013).

Ubiquitin conjugation to target proteins requires the action of 3 different enzymes. E1 (ubiquitin activating) enzymes activate and form thiolester bond with the ubiquitin molecule. Ubiquitin is then passed to an E2 by transthioylation. The E2(ubiquitin conjugating) enzyme along with the E3 (ubiquitin ligase) transfer the ubiquitin molecule to a lysine side chain of the target protein. E3 enzymes provide the target specificity for ubiquitination and are therefore the key regulator of ubiquitination (Hicke & Dunn 2003).



1.4 Ubiquitin conjugation to target proteins.

A) Activation of ubiquitin by E1 enzyme forming a high energy thioester bond. B) Ubiquitin is transferred to E2 conjugating enzyme by a transthioesterification reaction. C) Working together E2 and E3 enzymes form a thioester intermediate on the E3 ligase. D) Ubiquitin is transferred to target protein.

One fate of ubiquitinated proteins was degradation through the proteasome.

This is a large 26S multi-enzyme complex consisting of a central core 20S particle with two 19S particles capped on either side acting to recognise ubiquitinated substrates (Voges et al. 1999). Only substrates modified with a polyubiquitin chain of at least 4 molecules linked through lysine 48 (K48) are degraded in this manner (Acconcia et al. 2009; Ciechanover 2013). Ubiquitinated proteins are not only degraded and current evidence shows that many plasma membrane receptors and channels can be internalised due to ubiquitination (Urbé 2005). Furthermore the recycling and resensitization of the β_2 adrenergic receptor (Berthouze et al. 2009) and regulated surface expression of MHC II (major histocompatibility complex class II molecules) requires ubiquitination (Shin et al. 2006).

1.8 E3 ligases

As it has already been noted ubiquitin can direct trafficking of membrane proteins to degradative pathways. In the same way removal of ubiquitin by DUBs (deubiquitinating enzymes) can alter the trafficking of ubiquitinated proteins.

There are approximately 79 DUBs present in the human genome and they are responsible for the cleavage of ubiquitin molecules from proteins and therefore

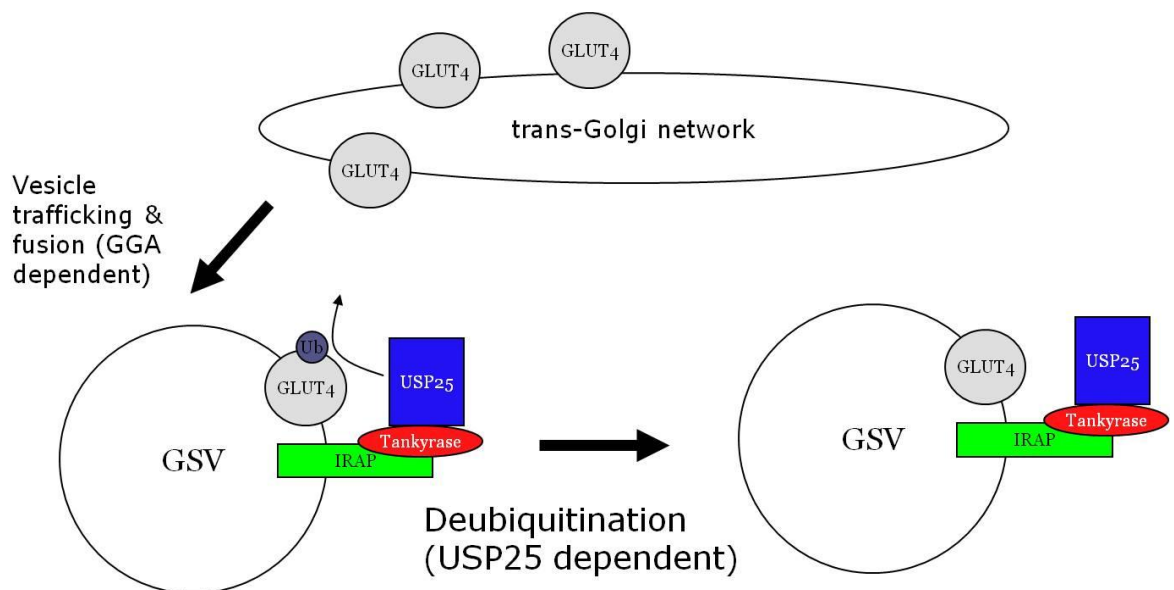
oppose the activity of E3 ligases (Komander et al. 2009). The DUBs have been sorted into five families; the ubiquitin C-terminal hydrolases (UCHs), ubiquitin-specific proteases (USPs), ovarian tumor proteases (OTUs), Josephins and JAB1/MPN/MOV34 metalloenzymes (JAMMs and MPN+)(Reyes-Turcu et al. 2009).

JAMM/MPN+ are zinc metalloproteases whereas the UCH, USP, OTU and Josephins are all cysteine proteases (Reyes-Turcu et al. 2009).

1.9 Hypothesis and aims

My current hypothesis is that GLUT4 requires ubiquitination and subsequent deubiquitination to enter and be stably maintained in GSVs. Figure 1.5 shows a mechanism for how this might be achieved. In this thesis I present experiments directed towards testing various aspects of this model. Namely I plan to investigate;

- A candidate E3 ligase that could be responsible for ubiquitination of GLUT4 and subsequent trafficking by GGA proteins to GSVs.
- I also intend to further test the evidence that USP25 catalytic activity is required for insulin responsive GLUT4 translocation.



1.5 Model of GLUT4 trafficking to GSVs and subsequent deubiquitination

2 Materials and Methods

2.1 Materials

2.1.1 Reagents, enzymes and media components.

All chemicals were from Sigma-Aldrich (Poole, Dorset, UK), VWR (Poole, Dorset, UK) or Fisher Scientific (Leicester, UK.) DNA restriction endonucleases, T4 DNA ligase and *Pfu*® polymerase were from Promega (Southampton, UK). Platinum *Pfx*® polymerase and dNTPs were from Invitrogen (Paisley, UK). Fast Start High Fidelity polymerase was from Roche diagnostics (Burgess Hill, West Sussex, UK). Broad range protein markers were from Biorad (Hertfordshire, UK). Bacterial media components were from Melford Laboratories Ltd (Suffolk, UK) and Formedium (Hunstanton, Norfolk, UK). Mammalian cell culture media components were from Invitrogen (Paisley, UK). Mirus TransIT TKO® transfection reagent was from Cambridge Bioscience Ltd (Cambridge, UK).

2.1.2 Bacterial strains

The bacterial strains used in this study are listed in Table 2.3 . Plasmids were maintained in XL-1Blue(Stratagene) or TOP10 (Invitrogen) *E.Coli*. Recombinant proteins were expressed in BL-21 (DE3) cells (Invitrogen).

2.2 Mammalian cell culture techniques

2.2.1 Cell culture of 3T3-L1 murine fibroblasts and adipocytes

3T3-L1 fibroblasts were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) newborn calf serum (NCS). Fibroblasts were maintained as subconfluent cultures at 37°C in a 10% CO₂ humidified incubator. The fibroblasts were grown to confluency and maintained for up to 3 days before induction of differentiation by addition of DMEM supplemented with 10% (v/v) foetal bovine serum (FBS), 0.25µM dexamethasone, 0.5mM 3-isobutyl-1-methylxanthine and 1µg/ml insulin. After 3 days, the differentiation mixture was replaced with DMEM containing 10% (v/v) FBS and 1µg/ml insulin. Adipocytes were re-fed with media containing 10% (v/v) FBS at 2 to 3 day

intervals and used for experiments between day 8 and day 12 post differentiation.

2.2.2 RNA oligonucleotide synthesis

RNA nucleotide synthesis in this study was carried out by Qiagen (Crawley, West Sussex) and Dharmacon (Lafayette, Colorado).

2.2.3 Transfection of 3T3-L1 adipocytes with siRNA oligonucleotides.

At day 6 and 7 post differentiation, 3T3-L1 adipocytes were incubated with siRNA oligonucleotides (details in table) complexed with TransIT-TKO® transfection reagent (Mirus, Madison, WI) using an adapted version of the manufacturer's protocol. For a 6 well plate, 5µl TransIT-TKO® and 5.3µl of 75mM stock siRNA solution was combined with 100µl serum-free DMEM per well. After 20 minutes, this mixture was added drop wise to individual wells of a 6 well plate. This process was scaled up for larger culture dishes. The siRNA treated adipocytes were then used for experiments on day 8 post differentiation.

2.2.4 Retroviral infection of 3T3-L1 fibroblasts

Fibroblasts were seeded to give approximately 40% confluency. The next day, 2ml of viral supernatant (produced as in (2.2.6) was combined with 2ml DMEM + 10% FBS, and 10µg/ml polybrene. This mixture was used to replace the fibroblast growth medium, and the cells were left to grow for 12 hours at 37°C. Cells were subsequently maintained in DMEM + 10% FCS with 2.5µg/ml puromycin to select for cells containing retrovirus. On confluency the cells were then differentiated as in (2.2.1) without drug selection, and then maintained in DMEM + 10% FBS with 2.5µg/ml puromycin until required for experiments.

2.2.5 Cell Culture of Plat-E cells

Plat-E cells were cultured in DMEM supplemented with 10% (v/v) FBS, 1µg/ml puromycin and 10µg/ml blasticidine. Cells were maintained as subconfluent cultures at 37°C in a humidified 5% CO₂ incubator.

2.2.6 Preparation of retrovirus using Plat-E cells

Cells were seeded at a density of 5×10^6 cells per 10 cm plate in DMEM + 10% FBS. On the following day, the cells were transfected with the appropriate retroviral constructs using Lipofectamine™2000. Following transfection, cells were maintained in DMEM + 10% FBS for 48 hours at 37°C, and subsequently switched to 32°C overnight to induce virus particle production. The viral supernatant was then harvested from the plates and stored at -80°C until required.

2.2.7 Use of crystal violet for counting viral plaques

For use in counting viral plaques for determining viral titres 1% crystal violet solution was prepared w/v in 20% ethanol. Virally infected cells were washed with 1ml of phosphate buffered saline and then incubated for at least 10 minutes with 1% crystal violet. The cells were then washed with 10% v/v ethanol and subsequent plaques identified and counted.

2.3 DNA manipulation

Standard DNA manipulation procedures were used throughout the study

2.3.1 Plasmid DNA purification

Promega Wizard® Plus SV miniprep kits were routinely used to isolate DNA from small bacterial cultures (<10ml). For larger preparations (>50ml) Qiagen® Plasmid Maxi kits were used

2.3.2 DNA oligonucleotide synthesis

DNA oligonucleotides were synthesised by Yorkshire Biosciences td (Heslington, York, UK) or IDT (Leuven, Belgium) and diluted in sterile double distilled water to a final concentration of 50pmol/μl before use.

2.3.3 Polymerase Chain Reaction

The high fidelity Platinum *Pfx*® polymerase (Invitrogen) was routinely used to PCR amplify desired DNA sequences.

A typical PCR mixture consisted of:

10mM dNTPs	5µl
50mM MgSO ₄	3µl
10x <i>Pfx</i> buffer	5µl
Enhancer solution	5µl
ddH ₂ O	28µl
Forward primer (50pmol/µl stock)	1µl
Reverse primer (50pmol/µl stock)	1µl
Plasmid DNA	1µl
<i>Pfx</i> DNA polymerase	1µl

And the conditions normally used were:

1.95°C	1 minute
2.94°C	90 seconds
3.56°C	1 minute
4.68°C	1minute/kb
5.68°C	10 minutes
6.4°C	Final hold

Steps 2 to 4 were repeated 30 times during the course of the PCR.

For amplification of larger templates and GC rich sequence the FastStart High Fidelity PCR system (Roche) was used. The mixture in this case consisted of:

10mM dNTPs	1µl
10x Reaction buffer	5µl
DMSO	1-5µl (2-10%)
Forward primer (50pmol/µl stock)	4µl
Reverse primer (50pmol/µl stock)	4µl
Template DNA	1µl (600ng)
2.5 U Fast Start High Fidelity enzyme	0.5µl
ddH ₂ O	to 50µl

For templates less than 3kb, a reaction profile essentially the same as that for Pfx was utilised. For longer template the following was used:

94° C	2 minute
94° C	30 seconds
52° C	30 minute
68° C	1minute/kb
94° C	30 seconds
52° C	30 seconds
68° c	1 min/kb (+ additional 20 seconds per cycle number)

68 °C 7 minutes

4 °C Final hold

Steps 2-4 were repeated ten times during the course of the reaction, followed by 25 cycles of steps 5-7.

Electrophoresis was used to resolve DNA fragments generated by PCR through 0.8% (w/v) agarose in tris-acetate (TAE) buffer (40mM Tris acetate, 1mM EDTA). DNA fragments were extracted from agarose gels using the QIAquick® gel extraction kit (Qiagen).

2.3.4 Site Directed Mutagenesis

Site directed mutagenesis (SDM) of DNA sequences was performed using Pfu® polymerase (Promega) according to the Stratgene QuikChange® method. Synthetic oligonucleotides, were used to exchange on amino acid residue for another with the mutagenic codon at the approximate centre of the oligonucleotide. A typical recipe for an SDM reaction is shown below:

Purified template plasmid	50ng
Forward primer	125ng
Reverse primer	125ng
10x Pfu Buffer	5µl
10mM dNTPs	1µl
Pfu Polymerase	1µl
ddH ₂ O	to 50µl

Typical reaction conditions were:

95 °C 1 minute

95 °C	50 seconds
60 °C	50 seconds
68 °C	16 minutes
68 °C	7 minutes
4 °C	Final Hold

Steps 2 to 4 were repeated 20 times during the course of the reaction. On completion of the reaction, the samples were treated with 1µl (10 units) *DpnI* for 1 hour at 37 °C, to digest methylated template DNA. 5µl of the final mixture was transformed into competent XL-1 Blue E.Coli, and transformants selected for on solid 2xYT (1.6% tryptone, 1% yeast extract, 0.5% NaCl) + 2% agar supplemented with 100µg/ml ampicillin. Several resultant colonies were picked, grown up in 10ml of 2xYT + 100µg/ml ampicillin and plasmid purified. Mutations were verified by DNA sequencing (Sequencing Service, University of Dundee).

2.4 Protein Methods

2.4.1 Electrophoretic separation of proteins

The separation of proteins by electrophoresis was carried out with discontinuous polyacrylamide gels (SDS-PAGE) following the basic method outlined by Laemmli (1970). Proteins were separated in gels composed of a stacking layer (5% acrylamide in a stacking buffer (0.25 M Tris-HCl (pH 6.8), 0.4% (w/v) SDS) and a separating layer (normally 10% acrylamide in separating buffer; 0.75 M Tris-HCl (pH 8.8), 0.4% (w/v) SDS). A 30% acrylamide-bisacrylamide mixture 37.5:1 ratio (Severn Biotech Ltd, Worcestershire) was used to make the gels. The tris-glycine electrophoresis buffer used contained 25 mM Tris-HCl, 250 mM glycine, 0.1%(w/v) SDS). Resolved proteins were visualised on gels by agitating the gels in Coomassie Brilliant Blue solution (0.25g Coomassie Brilliant Blue R250 in methanol: H₂O: glacial acetic acid (4.5:4.5:1 v/v/v)) for 30 minutes, followed by overnight agitation in destain solution (10% (v/v) methanol, 10% (v/v) glacial acetic acid) to give clearly visible bands.

2.4.2 Transfer of proteins to nitrocellulose membranes

A Bio-Rad Trans Blot® SD cell was used to transfer proteins from polyacrylamide gels onto nitrocellulose membranes (Protran, 0.45 µm pore). Gels and membranes were sandwiched between 6 pieces of Whatman 3M filter paper soaked in semi dry transfer buffer (50 mM Tris-HCl, 40 mM glycine, .037% (w/v) SDS, 10% methanol. The assembly was subject to a constant 180mA current for between 30 minutes (one gel) to one hour (three gels).

2.4.3 Immunoblot analysis

After protein transfer, unfilled site on the nitrocellulose membrane were blocked using 5% (w/v) non-fat dried milk in PBST 90.1% (v/v) Tween-20 in PBS) or TBST (0.1% (v/v) Tween-20 in Tris-HCl buffered saline (TBS - 0.5 M NaCl, 10m Tris-HCl pH7.6) according to the conditions required for each primary antibody. The membranes were exposed to primary antibody for 2 hours at room temperature or at 4°C overnight with agitation. Primary antibodies were diluted as described in table 2.1.

The membrane was subsequently washed six times with PBST/TBST for five minutes each. The membranes were exposed to secondary antibody for between 1 and 2 hours at room temperature, with agitation. A further six 5 minute washes in PBST/TBST were carried out and protein bands visualised using enhanced chemiluminescence (ECL) or LiCor.

2.4.4 Antibodies

Primary and secondary antibodies used in this study are detailed in table2.1. Polyclonal antisera against USP25 was generated by Eurogentec (Liege, Belgium) using an 87 day protocol. Briefly, two New Zealand white rabbits were immunised with 100µg recombinant USP25 at days 0, 14, 28 and 56 of the protocol. Serum was collected from the rabbits before the first immunisation (pre immune), and on days 38, 66 and 87 subsequently. The sera were stored at -80°C until use.

2.4.5 IgG Antibody purification

Sera collected from the final bleed on day 87 was combined 1:1 with phosphate buffered saline (PBS; 140 mM NaCl, 3 mM KCl, 1.5 mM KH₂PO₄, 8 mM Na₂HPO₄) plus protease inhibitor tablet (Roche) (containing leupeptin, α 2 macroglobulin, pefabloc SC, pepstatin, PMSF, chymostatin, E-64, bestatin, trypsin inhibitor). The subsequent mixture was incubated with 1ml of a 50% protein A-sepharose beads slurry with rotation for 1 hour. The beads were washed three times with cold PBS plus protease inhibitor cocktail (Roche). Elution from the beads was achieved by addition of 6x500 μ l elution buffer (50mM glutamine, pH2.5). The 6 fractions were then neutralised by addition of 1M Tris, pH10 and dialysed in PBS overnight.

2.4.6 Quantification of Immunoblots

Immunoblots were scanned using Phototshop® (Adobe) or transferred from Oddysey (LiCor) and immunoreactive bands measured using Image J software (National institutes of Health, USA). The integrated density of each band was measured and a background reading for an equally sized area of the image subtracted to give a numeric value for the band intensity.

2.4.7 GST fusion protein preparation

For productions of USP25-GST, 10 ml of 2YT + 100 μ g/ml ampicillin was inoculated with E. coli BL-21 (DE-3) cells harbouring a plasmid encoding the appropriate GST fusion protein and incubated at 37 °C overnight with shaking. The 10ml culture was inoculated into 400 ml Terrific Broth (1.2 % (w/v) tryptone, 2.4 % (w/v) yeast extract, 0.4 % (w/v) glycerol, 0.05 M K₂HPO₄, 0.016 M KH₂PO₄) and grown until OD₆₀₀ = 0.6. Protein expression was induced with 1mM IPTG and the cells were incubated for a further 4 hours at 37 °C with shaking. The cells were harvested by centrifugation and resuspended in 19.5 ml phosphate buffered saline (PBS; 140 mM NaCl, 3 mM KCl, 1.5 mM KH₂PO₄, 8 mM Na₂HPO₄). The cells were treated with lysozyme (1 mg/ml) on ice for 30 minutes and subsequently sonicated for five 30 second bursts with 30 second iced pauses. The lysate was spun at 17640 xg at 4 °C to remove insoluble components. The lysate was incubated with 1ml of a 50 % glutathione-Sepharose bead slurry with

rotation for 1 hour. The beads were washed three times with cold PBS to remove non-specifically bound proteins, and stored at 4 °C in PBS supplemented with EDTA free protease inhibitor cocktail (Roche) (containing leupeptin, α 2 macroglobulin, pefabloc SC, pepstatin, PMSF, chymostatin, E-64, bestatin, trypsin inhibitor) at a bead:buffer ratio of 1:1.

2.5 [³H]2-Deoxyglucose uptake assays

For these experiments cells were grown in 12 well plates. After differentiation cells were serum starved for two hours. Each twelve well plate was incubated at 37 °C and washed four times with Krebs Ringer Phosphate (KRP; 128 mM NaCl, 4.7 mM KCl, 5 mM NaH₂PO₄, 1.25 mM MgSO₄, 1.25 mM CaCl₂, pH7.4). The wells of each plate were treated as follows for 30 minutes at 37 °C:

- 3 wells 475 μ l KRP (Basal)
- 3 wells 475 μ l KRP + 1 mM insulin (Insulin)
- 3 wells 475 μ l KRP + cytochalasin B (Basal CytoB)
- 3 wells 475 μ l KRP + 1 mM insulin + cytochalasin B (Insulin CytoB)

After the 30 minute incubation, 25 μ l of [³H] deoxyglucose solution in KRP was added to the wells such that the final deoxyglucose concentration was 50 μ M with 0.25 μ Ci per well. After 5 minutes the contents of the wells was removed and the plates washed three times by immersion in ice cold PBS. Cells were air dried for 1 hour at room temperature and solubilised in 1 ml 1 % Triton X-100 for 2 hours. Solubilised cells were transferred into scintillation vials containing 5 ml scintillation fluid, and radioactivity associated with the cells measured using liquid scintillation spectrophotometry.

2.6 Indirect immunofluorescence

3T3-L1 fibroblasts were grown on sterilised coverslips before differentiation. Cells were starved of serum using serum-free DMEM for two hours prior to starting the procedure, and treated with or without insulin for 15 minutes prior to fixing. For surface epitope staining, cells were washed twice with PBS and fixed with 200 μ l 3 % paraformaldehyde (PFA) for 30 minutes, washed twice with PBS then washed twice with 20 mM glycine (GLY) in PBS to quench free aldehyde

groups. The coverslips were then incubated with 200 μ l blocking solution (2% BSA/20 mM glycine in PBS (BSA/GLY)) for 20 minutes. To incubate the cells with the primary antibody, the coverslips were placed cell side down onto a 40 μ l drop of primary antibody preparation (dissolved in BSA-GLY) on parafilm for 45 minutes, then washed four times with BSA/GLY. Secondary antibody in BSA/GLY was applied to the cells in a similar manner for 30 minutes and then washed as previously. The cells were returned to a 24 well plate, washed twice with PBS and surface bound antibodies fixed using 3 % PFA for 30 minutes. The coverslips were washed twice with PBS and the PFA quenched with GLY as previously. The cells were blocked and permeabilised using 200 μ l 20 mM glycine, 2 % BSA and 0.1% saponin in PBS (BSA/GLY/SAP) for twenty minutes. The coverslips were incubated with primary antibody preparation in BSA/GLY/SAP for 45 minutes, washed in BSA/GLY/SAP, incubated with secondary antibody for 30 minutes and given four further BSA/GLY/SAP washes. The coverslips were washed finally in PBS, dried and mounted onto slides using Immumount (Thermo). The mounted coverslips were analysed using a 63 x oil immersion objective lens fitted to a Zeiss LSM Pascal Exciter confocal fluorescence microscope, and images overlaid using LSM software (Zeiss).

Antigen	Working dilution	Description	Reference/Source
FAS	1:200 (IB)	Mouse monoclonal antibody against residues 9-202 of human fatty acid synthase.	BD biosciences.
GAPDH	1:30,000 (IB)	Mouse monoclonal antibody (clone 6C5) against GAPDH.	Ambion®
GLUT4	1:1000 (IB)	Rabbit polyclonal antibody against C-terminal 14 amino acids of GLUT4.	Synaptic Systems.
HA	1:1000 (IB) 1:200 (IF)	Mouse monoclonal antibody (clone 16B12) against an epitope from the human influenza haemagglutinin protein (YPYDVPDYA).	Covance Research products
Myc	1:1000 (IB) 1:500 (IF)	Rabbit polyclonal antibody raised against the myc epitope (EQKLISEEDL) conjugated to KLH.	Abcam
TrCP	1:500 (IB)	Rabbit polyclonal antibody raised against amino acids 93-384 of Human TrCP	Abcam
USP25	1:4000 (IB)	Rabbit polyclonal antibody raised against GST-USP25	(Bosch-Comas <i>et al.</i> , 2006)

2.1 Antibodies used in this study

Name	Target Sequence	Description
Scrambled	GACGAACAAACCGCCACATAT	Scrambled control siRNA
siRNA25	CCTGCTGGTTTAGTGCAAGTTA	Targeted against USP25 sequence obtained from Christopher Lamb PhD thesis
siRNA27 βTrCP	CCCAACGATCACTGCAAGAAA GTGGAATTTGTAGAACC	As above Purchased from Dharmacon®

2.2 siRNA oligonucleotides used in this study

Name	Genotype	Source
BL-21(DE3)	F ⁻ ompT hsdS _B (r _B ⁻ , m _B ⁻) gal dcm (DE3)	Invitrogen
TOP10	F ⁻ mcrA (mrr-hsdRMS-mcrBC) 80lacZM15 lacX74 recA1 ara139 (ara-leu)7697 galU galK rpsI (Str ^R) endA1 nupG	Invitrogen
XL-1 BLUE	recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac [F' proAB lacI ^q ZΔM15 Tn10(Tet ^r 0)]	Stratagene

Table 2.3 Bacterial strains used in this study

Chapter 3 β TrCP as a candidate E3 ligase

3

3.6 Introduction

Previous work carried out by our group has shown that GLUT4 is ubiquitinated in 3T3-L1 adipocytes (Lamb et al. 2010) and that ubiquitination is required for translocation of GLUT4 to the plasma membrane in response to insulin stimulation. Ubiquitination is carried out by E3 ubiquitin ligase enzymes that provide target specificity for ubiquitination and are therefore key regulators of ubiquitination (Ciechanover 2013). Previous work by (Govers et al. 1999) looked at ubiquitin dependent internalisation of growth hormone receptor and identified a novel ubiquitin conjugating motif. Designated as the ubiquitin dependent endocytosis (UbE) motif, it has been shown to interact with the F-box protein β TrCP (Beta-transducin repeat containing E3 ubiquitin ligase) that forms part of the Skp1, Cullin, F-box (SCF) E3 ligase complex. Other proteins suggested to contain a similar motif included GLUT4 and it was decided to investigate β TrCP as a candidate ligase for GLUT4 ubiquitination.

Two approaches were chosen to test whether β TrCP acts as the ubiquitin ligase responsible for conferring insulin sensitivity to trafficking of GLUT4. The first approach involved utilising retro viral expression of HA tagged GLUT4 constructs expressing Wt GLUT4 and GLUT4 V253A (a mutation to the proposed UbE motif) and see if this affected the ability of GLUT4 to translocate to the plasma membrane in response to insulin stimulation. The second approach utilised siRNA mediated knockdown of β TrCP directly to see if how this affected GLUT4.

3.7 Production of HA-GLUT4 V253A

Previous work by (Govers et al. 1999) identified the ubiquitin dependent endocytosis motif (UbE) and identified that GLUT4 may contain a similar motif. Utilising this information and with the help of Professor Gwyn Gould we identified a possible motif in GLUT4. Figure 3.1 shows the sequence of human and mouse GLUT4 aligned with the proposed UbE motif. Previous work by (Govers et al. 1999) also identified that mutation of phenylalanine 327 to alanine

in the UbE motif resulted in failure of growth hormone receptor to be internalised. To identify if a similar mutation could affect the ubiquitination of GLUT4 I produced a GLUT4 construct with a similar mutation. Our lab has previously used retro virally expressed HA-GLUT4 (pRM55) and I decided to use this as a template for producing a version of GLUT4 with its putative UbE motif mutated.

humanGlut4Seino	RKSLKRLTGWADVSGVLAELKDEKRKLERERPLSLLQLLGSRTHRQPLIIAVVLQLSQQL
300	
mouseGLut4	RKSLKRLTGWADVSDALAELKDEKRKLERERPM SLLQLLGSRTHRQPLIIAVVLQLSQQL
290	
UbEsequence	-----DSWVEF-----IELD-----
10	
	. * . : . * * .

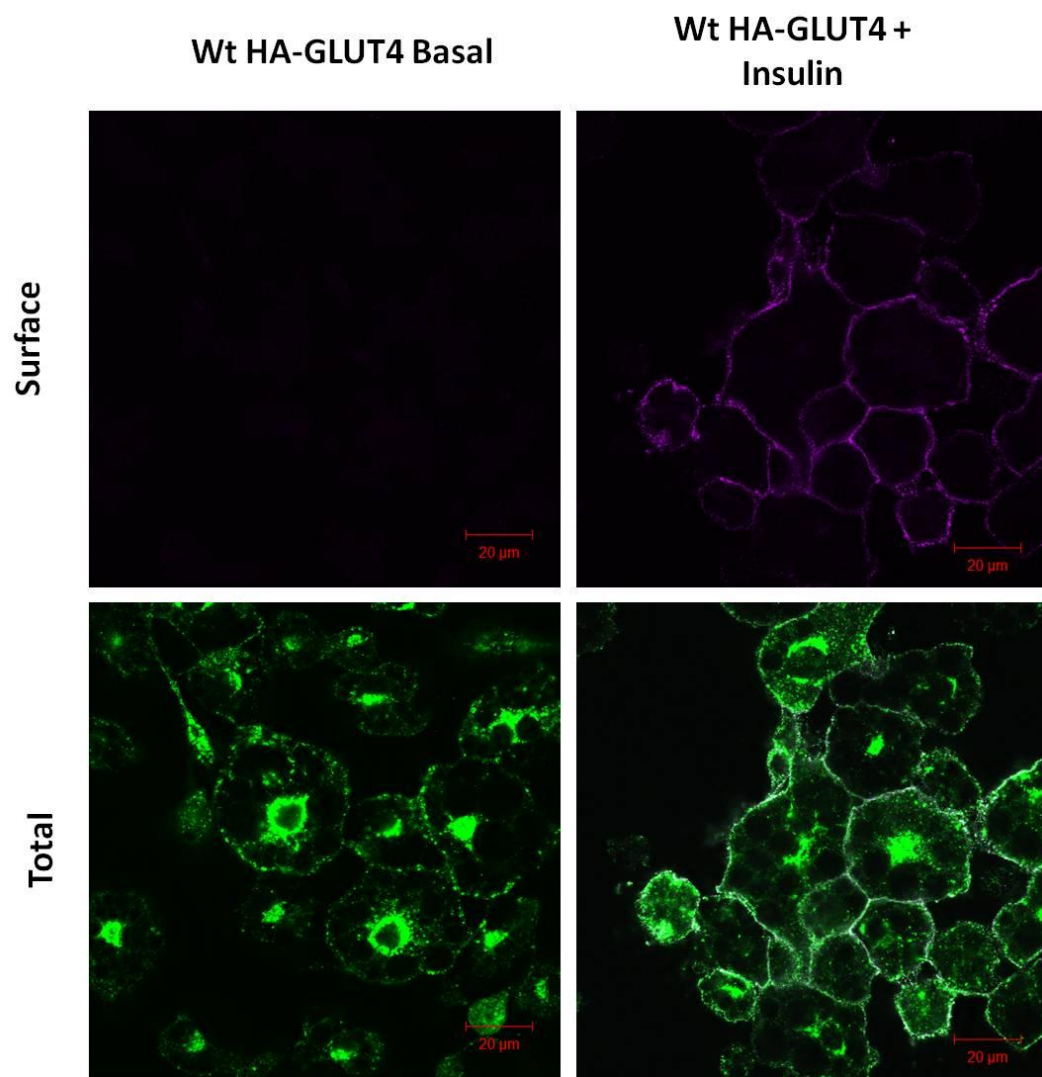
3.1 The UbE motif

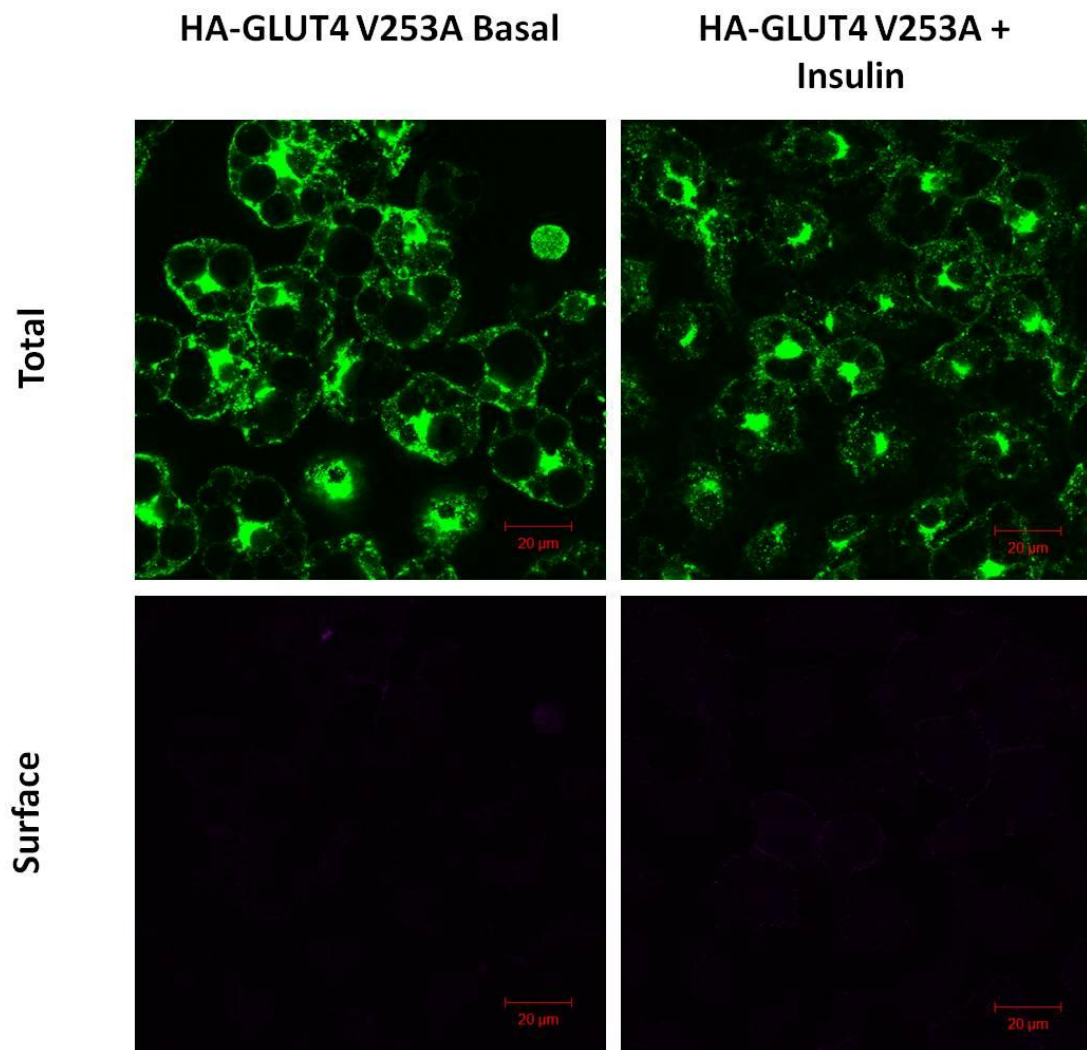
Alignment of Human and Mouse GLUT4 alongside the UbE motif, valine 253 is aligned with phenylalanine from the UbE motif.

Using pRM55 as the template and oligonucleotides designed to produce a valine to alanine mutation, HA-GLUT4 V253A (pISA2) was produced and confirmed by sequencing.

3.8 Expression of HA-GLUT4 V253A

With both the wild type GLUT4 and the UbE mutant now in retroviral expression vectors I was now able to compare translocation of these two constructs. The constructs were transfected into Plat-E cells to generate recombinant retrovirus and the virus was incubated with 3T3-L1 fibroblasts. The fibroblasts were grown on sterile coverslips where they were grown to confluency before subsequent differentiation. The adipocytes were then treated with or without insulin, before being fixed and stained for confocal microscopy. To differentiate from surface and total GLUT4 the cell were stained for the HA epitope prior to permeabilisation to detect HA-GLUT4 at the surface membrane and again after permeabilisation to show the total HA-GLUT4.





3.2 GLUT4 V253A fails to translocate in response to insulin

3T3-L1 fibroblasts were infected with retroviral constructs encoding HA-GLUT4 or HA-GLUT4 V253A, grown to confluency under selection with 2.5ug/ml puromycin and differentiated into adipocytes. Between days 8-12 post differentiation infected cells were treated with 170nM insulin for 20 minutes (insulin) or not (basal). Cells were fixed with 3% (v/v) paraformaldehyde and stained for surface (Purple) and total HA epitope (green). Scale bar = 20um

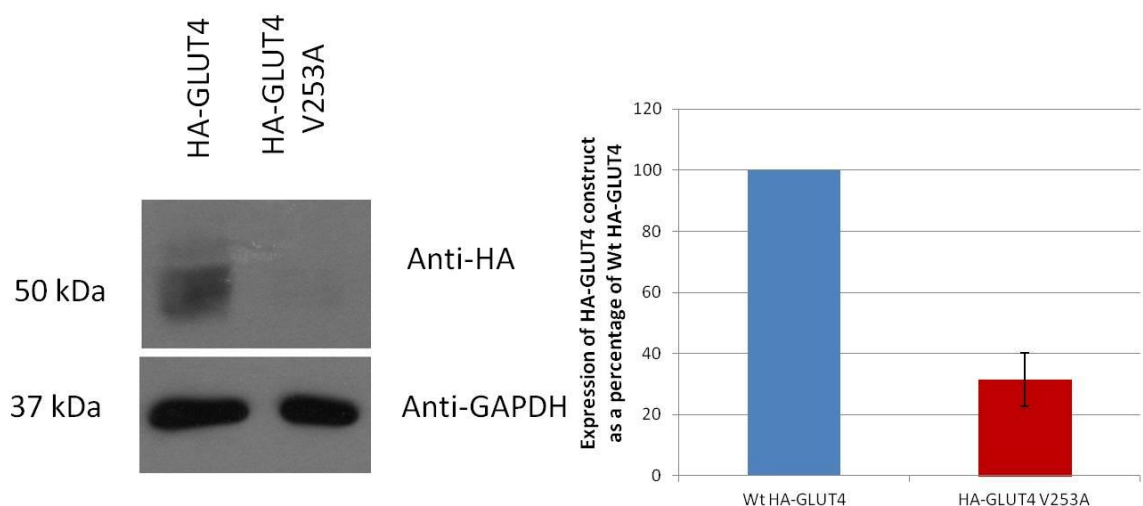
The above data suggest that the V253A mutant lacks the ability to translocate to the plasma membrane in response to insulin. The lack of surface staining (purple) present in the insulin treated cells indicates a failure to translocate to the plasma membrane. To better represent this data I carried out cell counts to look at the percentage of cells with each construct showing translocation to the plasma membrane. Three random fields of view were selected and a minimum of 50 cells per field were counted, cells were counted as showing surface staining if a defined membrane could be identified with the secondary surface stain (purple channel). As table 3.1 shows about 21% of cells expressing HA-GLUT4 V253A showed translocation to the plasma membrane compared to approximately 86% in cells expressing the wild type HA-GLUT4.

	Basal	Insulin
HA-GLUT4	0	86.18% ($\pm 1.24\%$)
HA-GLUT4 V253A	0	21.27% ($\pm 7.05\%$)

Table 3.1 Percentage translocation of HA-GLUT4 and HA-GLUT4 V253A in response to insulin.

A minimum of 50 cells from three fields of view were counted for presence or absence of cell surface HA staining. The results in the table are the mean of three experiments, expressed as the percentage of counted cells showing cells surface fluorescence \pm standard error of the mean.

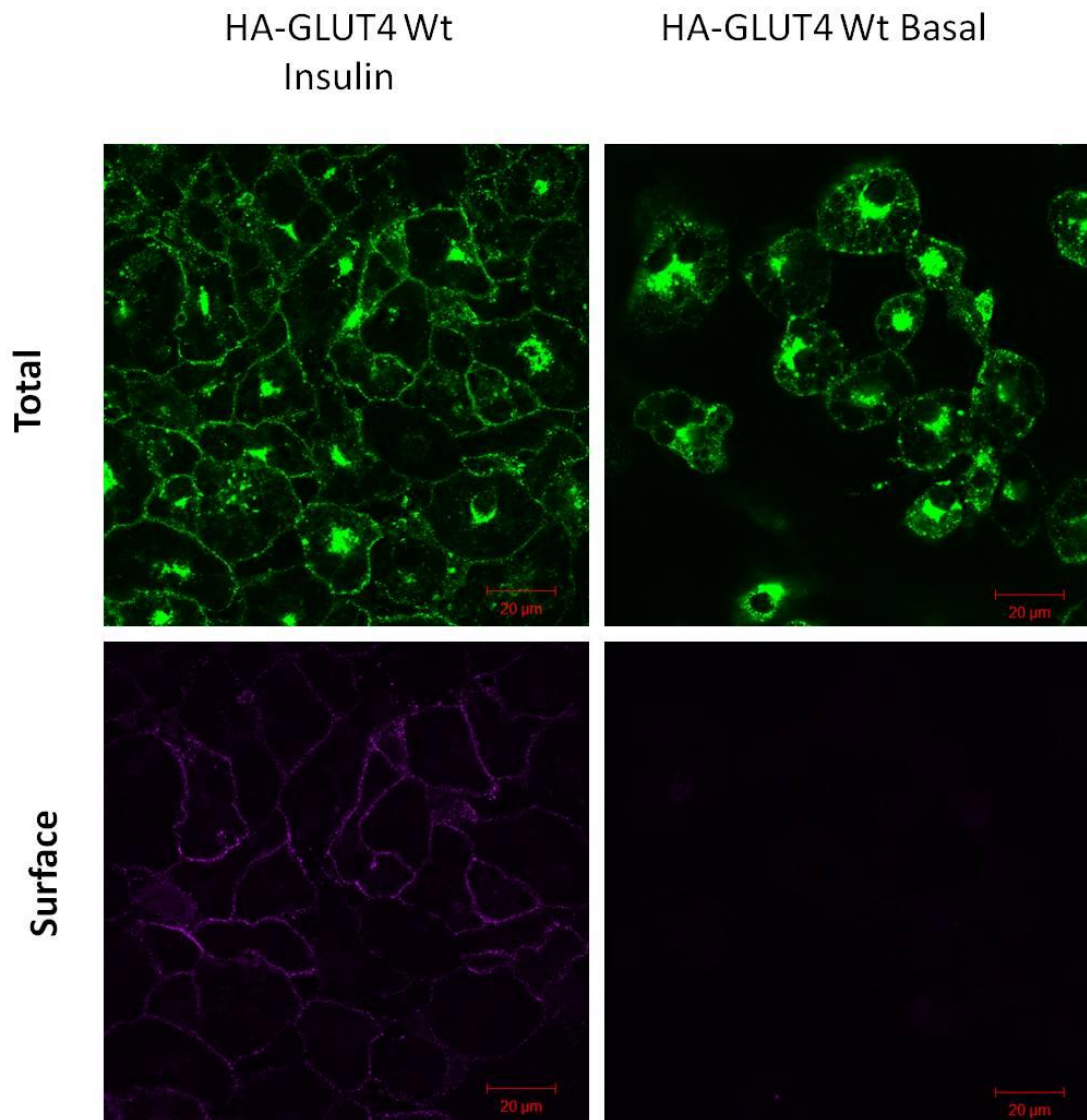
The immunofluorescence data in figure 3.2 suggests that both the wt HA-GLUT4 and the HA-GLUT4 V253A are expressed at similar level, to check the above data are not the result of different levels of expression of the constructs, plates of cells were grown in parallel to those grown on the coverslips. As with the coverslips infected 3T3-L1 adipocytes were grown to confluency before differentiation, lysates were produced and subsequently run on SDS-PAGE before analysis by western blotting. Figure 3.3 clearly shows that overall expression of GLUT4 V253A is reduced compared with the wild type HA-GLUT4 construct.

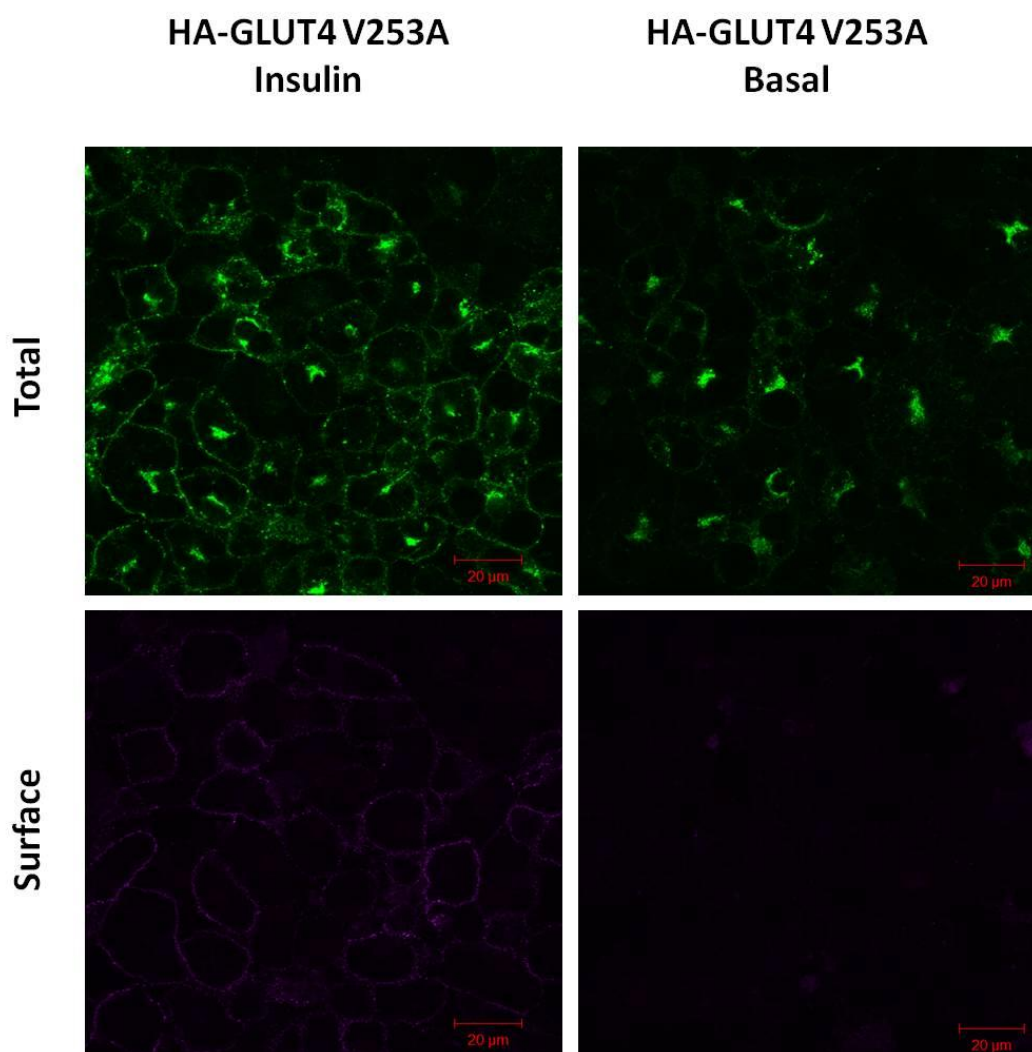


3.3 Viral expression of HA-GLUT4 V253A is lower compared to Wt HA-GLUT4.

3T3-L1 adipocytes were lysed in 50mM sodium HEPES, pH7.4, 150mM NaCl, 5mM EDTA, 1mM NEM, 1% v/v TRITON-X100 and protease inhibitors before homogenisation with 10 strokes with a needle and syringe. The lysates from these were centrifuged at 14000xg for 15 minutes and the supernatant removed from the pellet. Protein concentrations were estimated by micro BCA assay and 20ug were loaded on a 7.5% gel (v/v) and immunoblotted for the HA epitope and GAPDH. Error bars represent \pm standard error of the mean n=3

Given that the expressions levels are greatly reduced it is impossible to identify if the reduced translocation is due to the V253A mutation or just down to lower levels of HA-GLUT4. To address this I introduced the use of a crystal violet method for estimating viral titre. The use of crystal violet made viral plaques much more visible and prevented miscounting of plaques giving a better estimation of the viral titre. With this new system in place I proceeded to repeat the previous set of experiments.





3.4 Wt HA-GLUT4 and HA-GLUT4 V253A translocate to the plasma membrane in response to insulin.

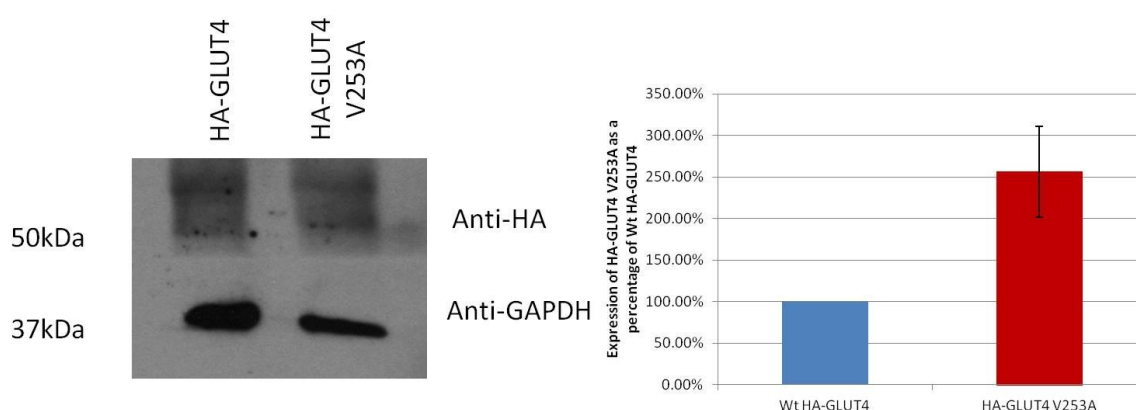
3T3-L1 fibroblasts were infected with retroviral constructs encoding HA-GLUT4 or HA-GLUT4 V253A, grown to confluency under selection with 2.5ug/ml puromycin and differentiated into adipocytes. Between days 8-12 post differentiation infected cells were treated with 170nM insulin for 20 minutes (insulin) or not (basal). Cells were fixed with 3% (v/v) paraformaldehyde and stained for surface (Blue) and total HA epitope (green). Scale bar = 20um

The data in figure 3.4 clearly indicates that HA-GLUT4 V253A translocates in a similar fashion to its wild type counterpart. This data is supported by the cell counts shown in table 3.2 as it shows over 80% translocation to the plasma membrane by both of the constructs. This would suggest that the lack of translocation observed in figure 3.2 was likely due to under expression of the V253A mutant. Expression levels of each construct were again checked using cells infected with the same batch of virus.

	Basal	Insulin
HA-GLUT4	0	82.25%(±6.48%)
HA-GLUT4-V253A	0.42%(±0.42%)	84.44%(±3.46%)

Table 3-2 Percentage translocation of Wt HA-GLUT4 and HA-GLUT4 V253A in response to insulin.

A minimum of 50 cells from three fields of view were counted for presence or absence of cell surface HA staining. The results in the table are the mean of three experiments, expressed as the percentage of counted cells showing cells surface fluorescence \pm standard error of the mean.



3.5 HA-GLUT4 V253A expression is increased compared to the wild type HA-GLUT4.

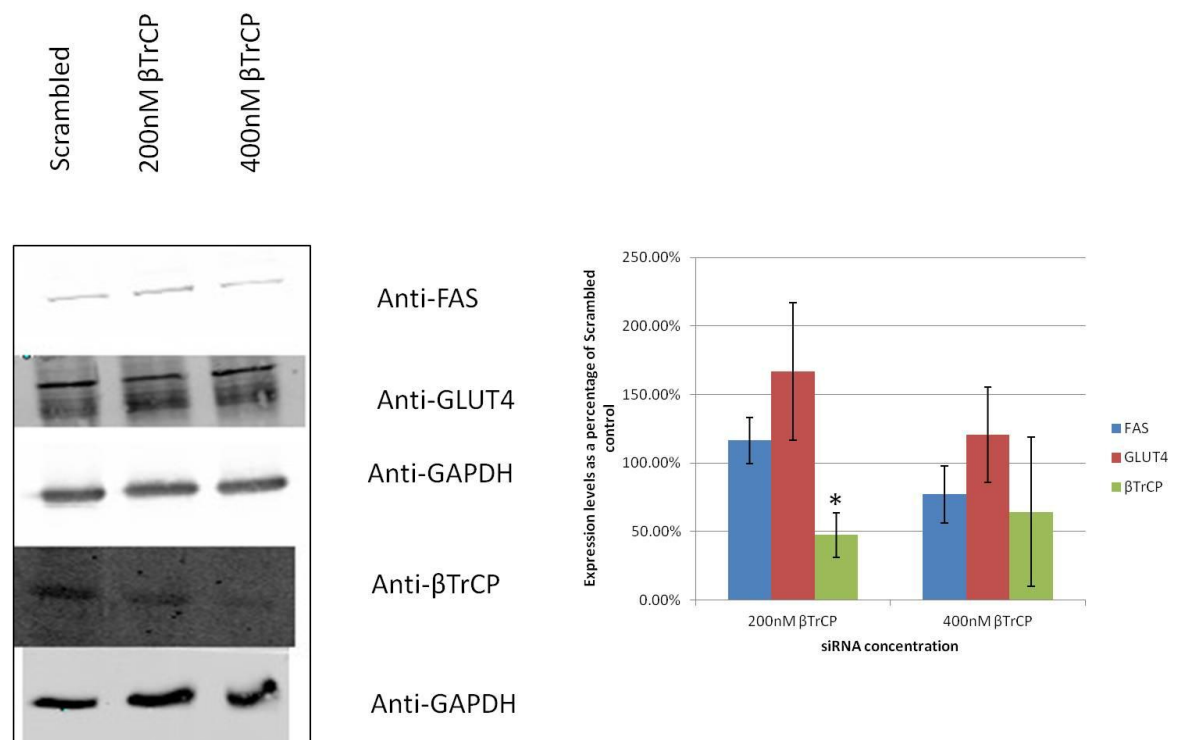
3T3-L1 adipocytes were lysed in 50mM sodium HEPES, pH7.4, 150mM NaCl, 5mM EDTA, 1mM NEM, 1% v/v TRITON-X100 and protease inhibitors before homogenisation with 10 strokes with a needle and syringe. The lysates from these were centrifuged at 14000xg for 15 minutes and the supernatant removed from the pellet. Protein concentrations were estimated by micro BCA assay and 20ug were loaded on a 7.5% gel (v/v) and immunoblotted for the HA epitope and GAPDH. Error bars represent \pm standard error of the mean n=3.

Controlling expression of the V253A mutant has been shown to be difficult as figure 3.5 clearly shows that even with equal amounts of virus HA-GLUT4 V253A is expressing much more than its' wild type counterpart. I attempted multiple times to achieve equal expression of these constructs with retroviral expression but was unable to produce two sets of virus that resulted in equal expression of the two constructs. As such no solid conclusions can be made from these data about the effect mutating the UbE motif has on translocation of GLUT4 to the plasma membrane.

3.9 siRNA mediated knockdown of β TrCP

To further investigate whether β TrCP plays a role in GLUT4 ubiquitination and subsequent trafficking to the insulin responsive compartment I decided to look

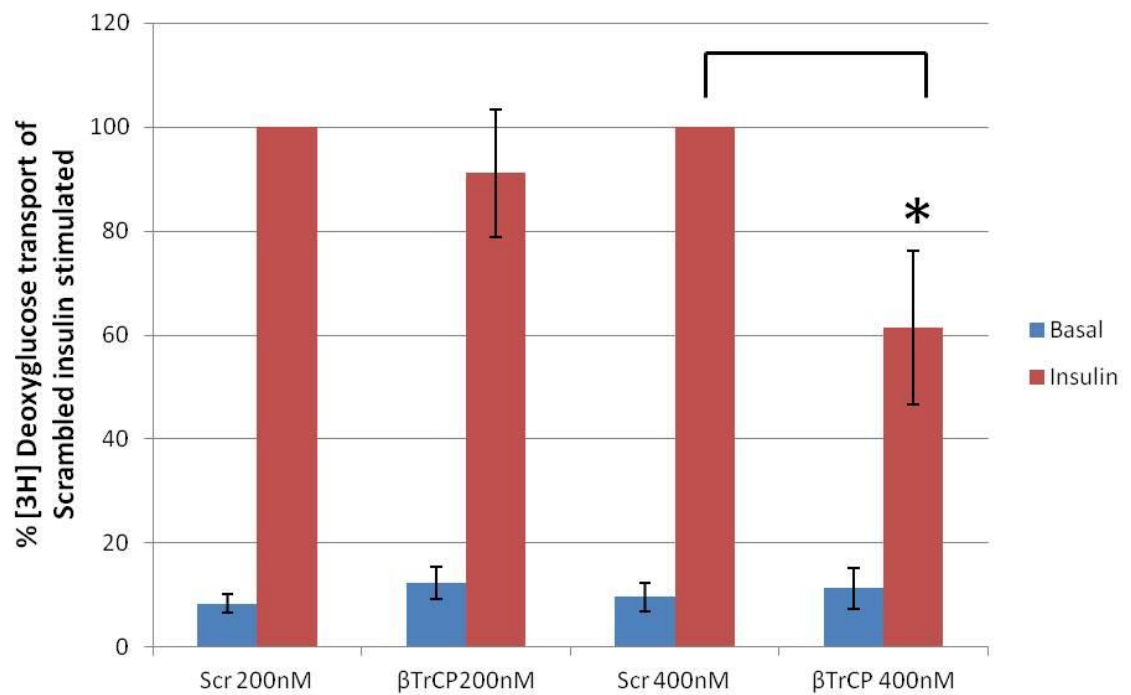
at the effects of knockdown of β TrCP in 3T3-L1 adipocytes. As the lab had previously used the Mirus TransIT TKO system to achieve siRNA-mediated knockdowns in 3T3-L1s and a β TrCP siRNA oligonucleotide was commercially available I decided to proceed using these tools to investigate if knockdown of β TrCP would reduce insulin responsive glucose uptake. Based on our model GLUT4 requires ubiquitination to enter GSVs and therefore knockdown of the E3 ligase responsible for ubiquitination of GLUT4 would result in a reduction of insulin responsive glucose transport.



3.6 siRNA mediated knockdown of β TrCP had no effect on GLUT4 expression levels.

3T3-L1 adipocytes were treated with the indicated concentrations of siRNA specific to β TrCP and lysates were subject to immunoblot analysis following separation on a 7.5% (v/v) SDS-PAGE gel. Expression levels of FAS, GLUT4 and β TrCP were analysed by image J software and analysed with an un-paired T-Test $^* = P < 0.05$. Error bars represent \pm standard error of the mean n=3.

The data in figure 3.6 show expression levels of GLUT4, FAS and β TrCP all normalised against GAPDH and then expressed as a percentage of the scrambled control. The right hand panel shows knockdown of β TrCP ranging from approximately 40-60% reduction in expression compared to the scrambled control. The data also indicates a trend towards increased expression of GLUT4 in these cells. This increase in GLUT4 levels however is not shown to be statistically significant. To identify if knockdown of β TrCP was altering insulin responsive glucose uptake I carried out deoxyglucose uptake assays with the siRNA treated 3T3-L1 adipocytes.



3.7 βTrCP reduces insulin responsive glucose uptake in 3T3-L1 adipocytes.

12-well plates of 3T3-L1 adipocytes were treated with the indicated concentrations of siRNA and assayed for uptake of [³H] deoxyglucose on day 8 post differentiation. Results were analysed using an unpaired student's T-test. *-p<0.05. Error bars represent ± standard error of the mean n≥3.

Figure 3.7 shows the uptake of [³H] deoxyglucose in 3T3-L1 adipocytes, treated with either scrambled siRNA or siRNA targeting βTrCP, under basal (blue) or insulin stimulated (red) conditions. These data indicate a significant reduction (approximately 40%) in deoxyglucose uptake in 3T3-L1 adipocytes treated with 400nm siRNA targeting βTrCP. A small reduction was also seen in cells treated with 200nm siRNA but was not shown to be significant. Across all repeats there appeared to be a trend for an increase in the basal transport of deoxyglucose, this was however not shown to be significant.

3.10 Discussion

The aim of the experiments in this chapter was to test βTrCP as a candidate E3 ligase for GLUT4 ubiquitination. To test this, I first looked at the UbE motif proposed to be present in GLUT4 (Govers et al 1999). Using site directed mutagenesis and an HA-GLUT4 retroviral construct already commonly used in our lab I was able to produce HA-GLUT4 V253A. Using indirect fluorescence microscopy I tried to identify if this mutation was altering GLUT4 trafficking in response to insulin stimulation in 3T3-L1 adipocytes. Initial experiments

appeared to show a reduction in surface staining in cells expressing HA-GLUT4 V253A (figure 3.2). This was shown to be due to reduced expression levels of the HA-GLUT4 V253A (figure 3.3). After discussion with other virus users in the lab I decided to implement use of crystal violet for counting viral plaques for estimating my viral titre, the rational being that human error was resulting in an un-even number of viral particles being used between my two constructs. After using this method and several repeats it became clear that getting equal expression of both constructs was proving to be incredibly difficult and it was decided to look at other ways of testing BTrCP as a candidate E3 ligase.

Having previously used siRNA knockdown of other proteins in 3T3-L1 adipocytes I decided to use this method to look more directly at BTrCP. With commercially available siRNA oligonucleotides and the transfection system already established in our lab I wanted to initially see if I could successfully lower expression of BTrCP in 3T3-L1 adipocytes. Initial attempts to lower expression of BTrCP using recommended concentrations of siRNA proved to show no effect on expression levels so I decided to try some higher concentrations of siRNA to see if I could produce knockdown. I eventually found that at concentrations of 200-400nM I was able to achieve significant knockdown (figure 3.6). As I did not observe any toxic effects of these levels of oligonucleotides in either the scrambled control or BTrCP treated cells I decided to continue with further experiments.

I wanted to address three key points regarding the BTrCP knockdown; did it have an effect on GLUT4 expression levels, did it alter insulin responsive glucose uptake and did it affect GLUT4 ubiquitination and ultimately translocation to GSVs. To address the first point I carried out immunoblot analysis of 3T3-L1 lysates that had been treated with BTrCP siRNA. I blotted all the samples for GLUT4, fatty acid synthase (FAS, as a marker of differentiation) and GAPDH as a loading control. After analysis with ImageJ software and an un-paired T-test there was shown to be no statistical difference in GLUT4 or FAS levels in the treated cells compared to the scrambled controls. These data would suggest that knockdown of BTrCP is having no effect on 3T3-L1 differentiation or on GLUT4 expression levels. Continuing with siRNA knockdown I decided to look at the effect on glucose uptake (figure 3.7) it became clear that BTrCP knockdown with 400nM siRNA resulted in a significant reduction in glucose uptake of about 40%. These data along with the fact GLUT4 levels are not reduced under BTrCP knockdown

could indicate that GLUT4 is not being targeted to the insulin responsive compartment. As BTrCP is an E3 ligase and we know that a mutant of GLUT4 that cannot be ubiquitinated does not respond to insulin it is possible that reducing levels of BTrCP results in GLUT4 failing to be ubiquitinated and in turn it is not targeted to the insulin responsive compartment.

To fully test this in future, ubiquitin pulldown of GLUT4 in BTrCP knockdown cells could be used to identify if GLUT4 is being ubiquitinated (Lamb et al. 2010). Another choice may be to look at another candidate E3 ligase such as Nedd4. The Nedd4-like ubiquitin ligase Rsp5p has been shown to be responsible for ubiquitination of Gap1p in *Saccharomyces cerevisiae* (Shiga et al. 2014) and as Gap1p and GLUT4 have been shown to have a similar trafficking pathways (Shewan et al. 2013) it would be a sensible target to investigate. Utilising similar knockdown experiments would help to identify if Nedd4 could play a role in ubiquitination of GLUT4.

4 Chapter 4

4.1 Introduction

Previous studies from our group have established that ubiquitination is required for entry of GLUT4 into GLUT4 storage vesicles (GSVs)(Lamb et al. 2010).

Ubiquitination alone however does not maintain GLUT4 in GSVs as ubiquitination of membrane proteins normally leads to degradation at the lysosome (Hicke & Dunn 2003; Urbé 2005) and, expression of a constitutively ubiquitinated version of GLUT4 (HA-GLUT4 7K/R-Ub) fails to translocate to the plasma membrane in response to insulin, and remains in the syntaxin 16 positive perinuclear compartment (McCann R.K. PhD thesis). Previous studies have also shown that only about 0.1% of GLUT4 is ubiquitinated (Lamb et al. 2010), and looking at these data together suggests that ubiquitination of GLUT4 is transient and therefore requires a deubiquitination step.

Deubiquitinating enzymes (DUBs) function to maintain free ubiquitin levels and to promote stability of ubiquitinated substrates (Komander et al. 2009).

Receptor recycling and resensitisation in the endosomal system has been shown to require DUBs; internalisation of the $\beta 2$ adrenergic receptor requires ubiquitination of the receptor and subsequent deubiquitination by ubiquitin specific protease (USP) 20 and 33 (Berthouze et al. 2009). Ubiquitination of the receptor triggers internalisation upon ligand binding and targets it toward the lysosome, deubiquitination of the receptor rescues it from degradation and returns it to the cells surface for subsequent stimulation and internalisation. Previous studies in our lab and from others suggest that USP25 may be the DUB acting to deubiquitinate GLUT4 and maintain GLUT4 stability in GSVs.

The insulin responsive amino peptidase IRAP is one of the main cargo components of GSVs, and traffics in an identical manner to GLUT4 under both basal and insulin stimulated conditions (Ross et al. 1997b). Depletion of IRAP utilising siRNA in 3T3-L1 adipocytes has been shown to reduce insulin stimulated translocation of GLUT4 however depletion of GLUT4 does not seem to have a similar effect on IRAP (Yeh et al. 2007). These data suggest IRAP is involved in maintaining GLUT4 in GSVs and provides a link to USP25. IRAP has been shown to interact with the poly-ADP ribose polymerase (PARP) Tankyrase, via a minimal

tankyrase binding motif (RXX(P,A)DG) (Chi & Lodish 2000), and tankyrase has been shown to interact with the DUB USP25 through yeast two hybrid analysis (Sbodio & Chi 2002). Further work carried out in our lab (Lamb, C thesis) used in vitro pulldown to show that tankyrase from 3T3-L1 adipocytes interacts with GST-USP25 but not with GST-USP25 R1049A which contains a mutation in the previously identified Tankyrase binding site (Chi & Lodish 2000).

siRNA depletion of USP25 in 3T3-L1 adipocytes has been shown to result in lower expression levels of GLUT4 and to reduce insulin stimulated glucose uptake. Taken together these data suggest a model where IRAP and tankyrase form a scaffold on GSVs that allows USP25 to bind and deubiquitinate GLUT4. This cycling of ubiquitination and deubiquitination would allow GLUT4 to enter and be retained in GSVs. Reduction of USP25 expression through siRNA knockdown prevents deubiquitination of GLUT4 and GLUT4 is transported to the lysosome and degraded.

4.2 Aims of the chapter

The overall aim of the work presented in this chapter was to further test the model outlined above. To this end, I investigated whether GLUT4 and USP25 colocalise in 3T3-L1 adipocytes. I also set out to characterise the role of the deubiquitinating enzyme USP25 in GLUT4 sorting. Specifically I wanted to ask whether USP25's deubiquitinase activity is required to stabilise GLUT4 in GSVs. I aimed to address this by expressing siRNA resistant USP25, and a catalytically-inactive version thereof, in USP25 depleted cells, and comparing their abilities to rescue the observed reduced levels of GLUT4 and insulin-responsive glucose uptake.

To pursue the above aims, I first embarked on a series of 'tool building' to gather the necessary reagents.

4.3 Affinity purification of USP25 antisera

Utilising the human USP25 sequence published in (Bosch-Comas et al. 2006), our group generated USP25 antisera that could detect USP25 in adipocyte lysate (Christopher Lamb PhD thesis). In addition to recognising USP25, this antisera

also recognises a number of other proteins in adipocyte lysates. To address this issue I set out to clean up the antibody for use in further experiments. As the final bleed had been shown to detect USP25 to a greater degree than other available sera I chose this as my starting material. Purification of antibodies was carried out as detailed in section 2.4.5 and the resulting material was used in immunoblot analysis of adipocyte lysates (Figure 4.1). To characterise the purified antibody, I used it at a 1:1000 dilution in immunoblot analysis of lysates prepared from 3T3-L1 adipocytes. The left hand panel of Figure 4.1 shows that the purified antibody recognises several bands in the adipocyte lysates (although not as many as seen with the unpurified sera; Christopher Lamb, PhD thesis). To investigate which, if any, of these are specific to USP25, I ran a second immunoblot analysis in parallel, with the same lysates but this time probed with 1:1000 of the purified antibody in the presence of 4 μ g/ml of GST-USP25 (right hand panel of Figure 4.1).

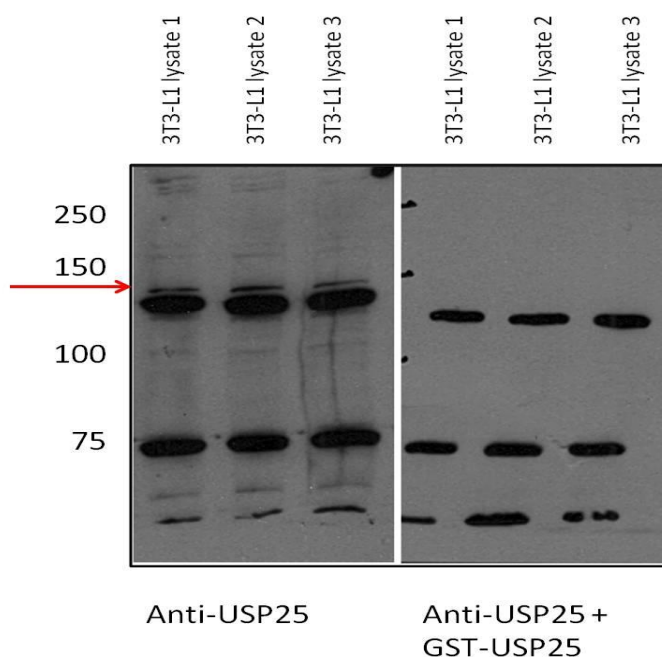


Figure 4.1 Characterisation of anti-USP25 antibodies

Lysates (20 μ g protein) prepared from 3 independent passages of 3T3-L1 adipocytes were subject to immunoblot analysis following separation on a 7.5% (v/v) SDS-PAGE gel. The two panels shown represent identical blots with the left hand panel being probed with purified USP25 antibody and the right hand panel being probed with the same in the presence of 4 μ g/ml recombinant GST-USP25.

A band of approximately 140kD (the observed molecular weight of USP25; (Meulmeester et al. 2008) can be seen in all lanes of the left hand panel (indicated by the red arrow), but is missing from the right hand panel. Given that the only difference between this is the inclusion of GST-USP25 in the primary antibody step, this band is likely to represent USP25.

4.4 Identification of suitable siRNA for USP25 knockdown

With a suitable antibody available to detect USP25 for western blotting I then needed to identify a suitable method for depletion of endogenous USP25 for use in further experiments. A well established method of depleting proteins of interest without having to knock out the gene of interest is RNA interference (RNAi) (Elbashir et al. 2001). To this end I used the Mirus TransIT-TKO® transfections system (detailed 2.2.3) and two oligonucleotides (siRNA25 and siRNA27 detailed table 2.2) already available to our lab. 3T3-L1 adipocytes were transfected with 50nM siRNA on days 6 and 7 post differentiation and harvested on day 8. Lysates were run on SDS-PAGE prior to immunoblot analysis utilising the previously characterised USP25 antibody. The immunoblots were quantified utilising ImageJ software by normalising intensities of USP25 against a band of unknown origin at 75kDa the results are shown in figure 4.2.

These data indicate that USP25 is depleted by both siRNA25 and siRNA27 when compared to the scrambled siRNA control $54\% \pm 20\%$ and $27\% \pm 2.5\%$ respectively. These data indicates that siRNA27 provided a more robust and consistent knockdown of USP25 and I therefore decided it would be used in future experiments.

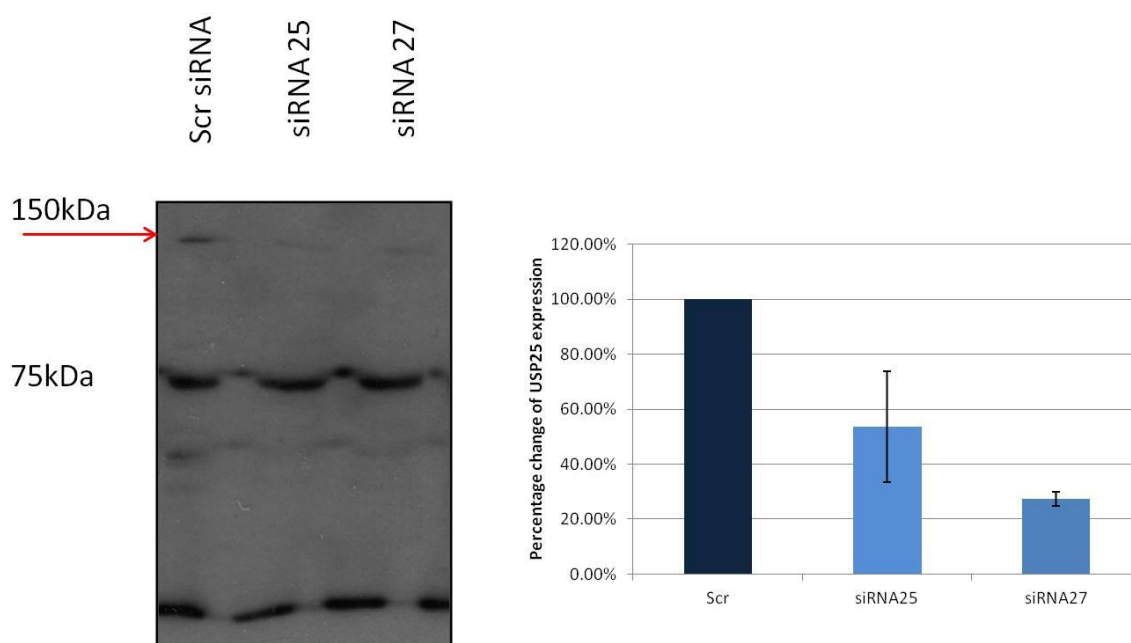


Figure 4.2 Immunoblot analysis of 3T3-L1 adipocytes treated with siRNA specific to USP25 shows a reduction in USP25 levels

(Left panel) 3T3-L1 adipocytes were treated with 50nM of the indicated siRNA using Mirus TransIT TKO transfection reagent on days 6 and 7 post differentiation, harvested on day 8 and immunoblotted for USP25. (Right panel) Levels of USP 25 (band indicated by red arrow) were analysed using imageJ software and normalised against the band of unknown origin at approximately 75 kDa.

4.5 Production of tuneable myc-USP25 and myc-USP25 C178S

Work carried out previously in our lab has shown that using a retro-viral method to express USP25 in 3T3-L1 adipocytes prevents them from successfully differentiating. To overcome this problem I employed an inducible virus system that would allow expression of USP25 constructs to be triggered post differentiation. I chose to utilise the Proteo Tuner™ system which allows for titratable expression of the desired protein by controlling the concentration of the induction reagent Shield1. The pRetroX tuner plasmid expresses a destabilisation domain attached to your protein of interest leading to rapid degradation under normal circumstances. When Shield1 is added it binds to the destabilisation domain and prevents it from being recognised. With the destabilisation domain blocked your protein of interest is then expressed, and this expression is titratable by altering the concentration of Shield1 used.

Polymerase chain reaction was used to produce a USP25 product encoding myc tagged human USP25 and was flanked by NotI and BAMHI restriction endonuclease sites. The resultant product was sub-cloned into the pCR2.1 vector containing NotI and BAMHI restrictions sites. After sub-cloning into pCR2.1 site directed mutagenesis was used to produce a product encoding USP25 C178S a catalytically inactive mutant (Denuc et al. 2009). I used pCR2.1 as my vector for the SDM as it already contained the NotI and BAMHI restrictions sites I required for sub-cloning into the pRetroX-Tuner vector. Utilising theses restriction sites the wild type USP25 and the USP25 C178S were ligated into the pRetroX Tuner vector to be used in viral production. Both constructs were transfected into Plat-E packaging cells to produce recombinant retrovirus as described in 2.2.6. The platinum-E packaging cells express viral coat proteins not expressed in the pRetroX vector and as such produce a virus that can infect cells but cannot produce and infectious virus in the infected cell line. Sub confluent 3T3-L1 fibroblasts were infected with the resulting product, grown to confluency and differentiated into adipocytes. Infected adipocytes were treated for 24 hours with varying concentrations of Shield1 and subsequent lysates were immunoblotted for myc-USP25 (figure4.3). Initial experiments showed that only the USP25 C178S was expressed upon Shield1 induction.

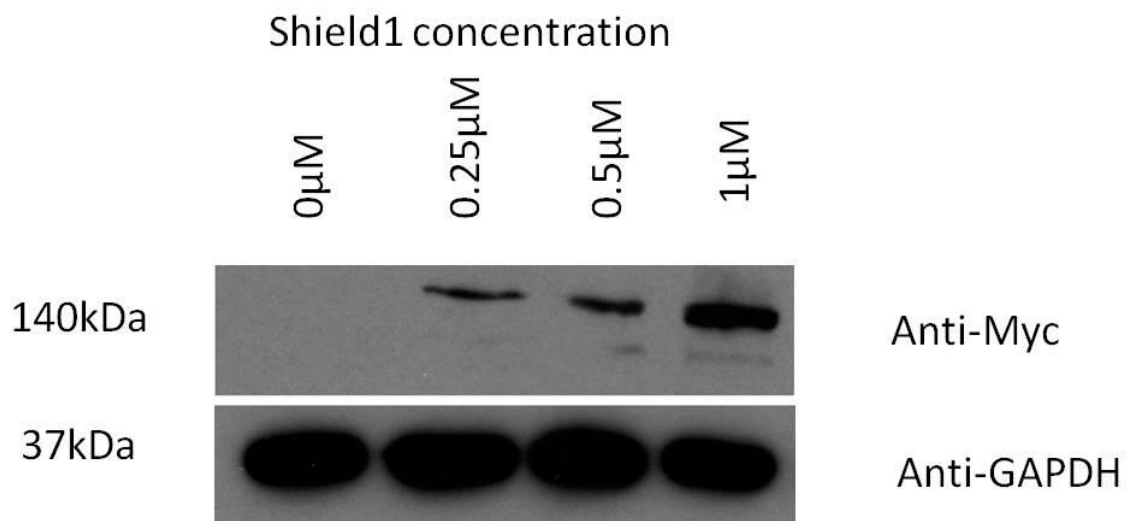


Figure 4.3 myc USP25 C178S expression increases as Shield1 concentration increases
3T3-L1 fibroblasts were infected with retroviral constructs encoding Myc-USP25 C178S. Between days 8-12 post differentiation infected cells were treated for 24 hours with the Shield1 reagent in a concentration range from 0.25μM-1μM. Infected adipocytes were subject to immunoblot analysis following separation on a 7.5% (v/v) SDS-PAGE

As both sequences had been sequenced prior to viral production, and were shown to be correct, it was assumed that the initial viral batch of Wt USP25 had

failed to infect the 3T3-L1 adipocytes. After multiple attempts to produce virus that would express wild type USP25 it was decided to use USP25 C178S as a template (as we knew it expressed under Shield1 induction) and use SDM to get back to wild type USP25. This method appeared to be successful as Figure 4.4 shows expression of wt USP25 under Shield1 induction.

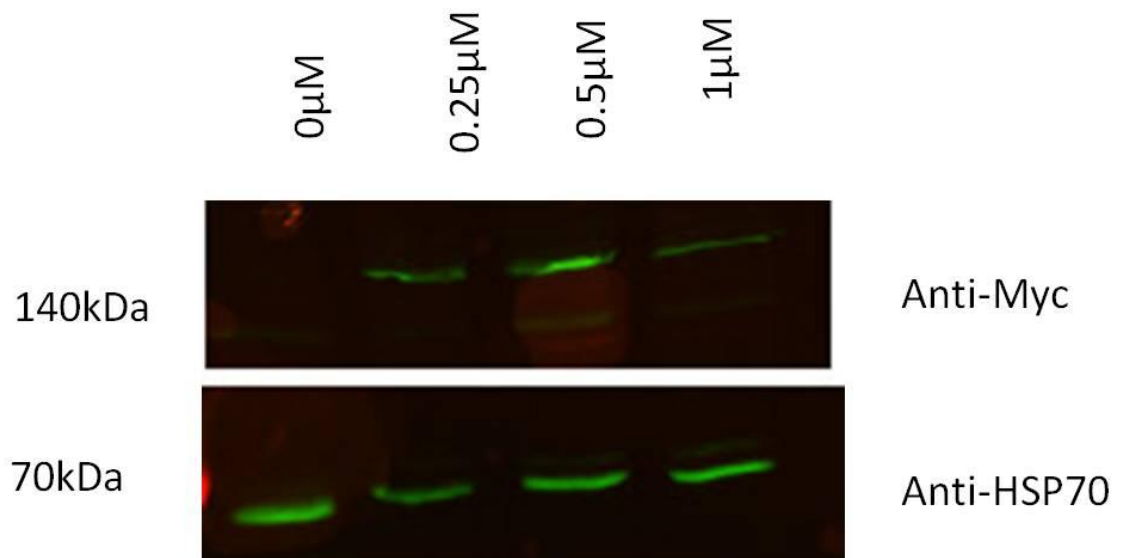


Figure 4.4 Wt USP25 expression increases as Shield1 concentration increases

3T3-L1 fibroblasts were infected with retroviral constructs encoding Myc-USP25. Between days 8-12 post differentiation infected cells were treated for 24 hours with the Shield1 reagent in a concentration range from 0.25μM-1μM. Infected adipocytes were subject to immunoblot analysis following separation on a 7.5% (v/v) SDS-PAGE.

4.6 Discussion

The aim of this chapter was to further characterise the interaction between GLUT4 and USP25. To address this I set out on a series of tool building exercise. To allow for detection of USP25 by western blotting I set out to purify a previously available antibody. As figure 4.1 shows a distinct band representing USP25 can be detected from 3T3-L1 adipocyte lysate. With that success I then wanted to look at ways to express USP25 constructs in 3T3-L1 cells without inhibiting differentiation. Utilising an inducible virus system I was able to express USP25 and USP25 C178S post differentiation (figure 4.3 and 4.4). This system however proved unreliable as repeats even using virus produced from the same batch were unable to produce consistent results. This method does show that USP25 can be expressed post differentiation in 3T3-L1 adipocytes and if this work was continued with a more robust system it could be used to further characterise the interaction between GLUT4 and USP25.

To address this infectivity problem in the future a lentiviral system could be used instead of the current retroviral system. Lentivirus has previously been shown to be successful at infecting adipocytes and pre-adipocytes (Carlotti et al. 2004) furthermore a proteo tuner inducible system of expression is available as a lentiviral vector. Use of an inducible lentiviral system should allow expression of USP25 constructs reliably without inhibiting differentiation. With this system in place it would be possible to identify if the catalytic activity of USP25 is required for GLUT4 to be maintained stably in GSVs.

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